JOURNAL OF THE NATIONAL CANCER INSTITUTE

August 1955
Volume 16 • Number 1

A Publication of the
U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

JOURNAL OF THE NATIONAL CANCER INSTITUTE

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JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • Public Health Service

Volume 16
Number 1

August 1955

Recovery from Acute Radiation Injury in Mice Following Administration of Rat Bone Marrow[1, 2]

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It has been established by experimental work from several different laboratories that the acute effects of total-body X irradiation, at lethal dose levels, can be modified by certain procedures carried out after radiation exposure. Thus, intraperitoneal implantation of spleens from immature mice (*1*), intravenous or intraperitoneal injection of bone-marrow suspensions (*2*), and intraperitoneal or intravenous administration of splenic-tissue homogenates (*3*) have been shown to afford protection against acute radiation death at lethal and immediately supralethal radiation dose levels. The bulk of such studies have been carried out with homologous tissue preparations, for example, bone marrow from nonirradiated LAF_1 mice injected into irradiated LAF_1 mice. The degree of protection afforded by these biological materials appears to be modified by genetic factors (*4*). In general, bone marrow and spleen preparations are effective when the recipient irradiated animals are of the same species and strain as that from which the tissue material is obtained (*5*, *6*). However, recent experimental observations by Congdon and Lorenz (*7*) indicate that the species specificity of bone marrow for radiation protection is not absolute. A significant percentage of irradiated strain LAF_1 and A mice (800 r and 900 r total-body X irradiation) were protected against radiation death (21-day endpoint) by a single intravenous injection of rat bone-marrow suspension. In view of the implications of heterologous postprotection studies for the question of the mechanism(s) of action of the protective principle in bone marrow, it was considered desirable to extend the observations of Congdon and Lorenz. It was of particular interest to determine whether irradiated mice receiving rat bone marrow would survive the 30-day observation period, and to ascertain whether recovery of hematopoietic tissue was elicited by the marrow treatment.

[1] Received for publication November 22, 1954.

[2] This work was supported, in part, by funds from the Bureau of Medicine and Surgery, U. S. Navy Department.

[3] The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official, or as reflecting the views of the Navy Department.

[4] The authors are indebted to Dr. Rachael K. Reed for the histopathologic data on the bone-marrow sections, and to Patricia L. Roan for preparation of the histologic specimens.

Materials and Methods

Female LAF_1 mice 14 to 16 weeks of age were used in this study. In each experiment up to 40 mice were exposed at one time to total-body X irradiation (250 KVP), at doses of 790 r to 815 r. This dose range is uniformly fatal to 100 percent of untreated mice within 14 days. The radiation factors and the exposure procedure were the same as those used previously (*3*). Following irradiation, the mice were transferred to metal cages, 10 to a cage, and given Purina laboratory chow and tap water *ad libitum*.

The bone-marrow donors were male and female Sprague-Dawley rats ranging in age from 11 to 35 weeks. The marrow was collected by aspiration from the femurs into a tared weighing bottle, as described in a previous report (*8*), weighed, and suspended in either of two suspending media—*M*/15 phosphate buffer (*p*H 7.0), containing 5×10^{-4} *M* adenosinetriphosphate (ATP) and 1 mg. per ml. dextrose, or sucrose-salt-ATP medium (*9*). A 0.2-ml. volume of the chilled marrow suspension (equivalent to 60–90 mg. of rat bone marrow) was administered by tail vein to each of the irradiated mice usually within 2 or 3 hours after exposure to radiation. The animals were not anesthetized during injection. An equivalent volume of buffer or of sucrose-salt solution was injected into each of the control mice.

Several criteria for evaluating the effectiveness of heterologous bone-marrow protection were employed: body-weight changes, peripheral leukocyte levels, spleen DNA content (*10*), and histologic appearance of the bone marrow.

In several experiments, the irradiated mice were injected with cortisone in conjunction with bone marrow. A saline suspension of cortisone acetate (Cortone, Merck) was used, each mouse receiving 1.25 mg. of cortisone per injection intraperitoneally.

Results

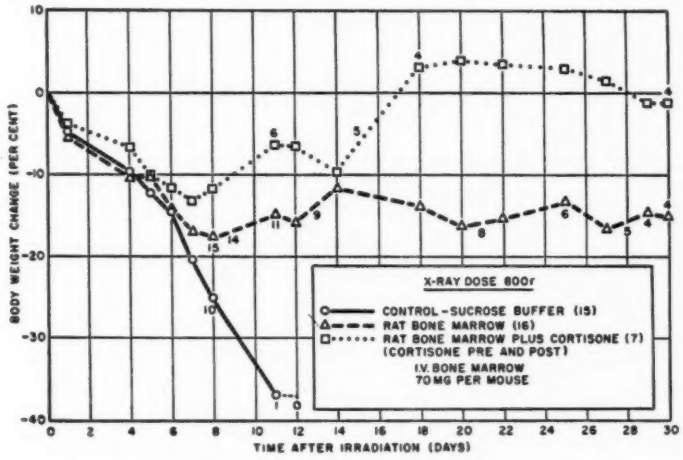
The experimental data, summarized in table 1, show the 30-day survival observed in four separate experiments in which X-irradiated mice received a single intravenous injection of rat bone-marrow suspension (approximately 60 mg. wet weight). Under these conditions, up to 50 percent of the treated mice were alive 30 days after irradiation, whereas all of the control irradiated mice were dead by the 14th day.

Recovery of the irradiated mice treated with rat bone marrow was reflected in the cessation of loss in body weight by the 8th day post-irradiation, whereas, by contrast, the control irradiated mice, injected with buffer solution, continued to lose weight until death (text-fig. 1). Leukocyte counts on tail-vein blood indicated partial recovery of hematopoiesis by the 12th day in the marrow-treated mice. At this time the blood contained 1,000 granulocytes and 300 lymphocytes per cu. mm.; at 18 days 4,800 granulocytes and 3,000 lymphocytes; at 21 days 6,000 granulocytes and 4,500 lymphocytes. The control irradiated mice, on the other hand, had a total leukocyte level of approximately 350 cells per cu. mm. on day 12.

TABLE 1.—*Modification of radiosensitivity of mice by rat bone-marrow injection*

Expt. No.	X-ray dose (*r*)	Treatment	30-Day survival		Mean survival time* (*days*)
			No./total	Percent	
1	790	Rat bone marrow (75–90 mg.)	5/21	24	10. 6
	790	Phosphate buffer	0/9	0	9. 2
2	800	Rat bone marrow (73 mg.)	3/13	23	14. 5
	800	Rat bone marrow (73 mg.) †	1/6	17	17. 3
	800	Phosphate buffer	0/11	0	8. 7
3	800	Rat bone marrow (63–76 mg.)	4/8	50	15. 2
	800	Phosphate buffer	0/8	0	9. 6
4	800	Rat bone marrow (63 mg.)	5/16	31	7. 8
	800	Phosphate buffer	0/9	0	8. 8
5	800	Rat bone marrow (70 mg.)	5/15	33	19. 6
	800	Sucrose buffer	0/9	0	10. 3

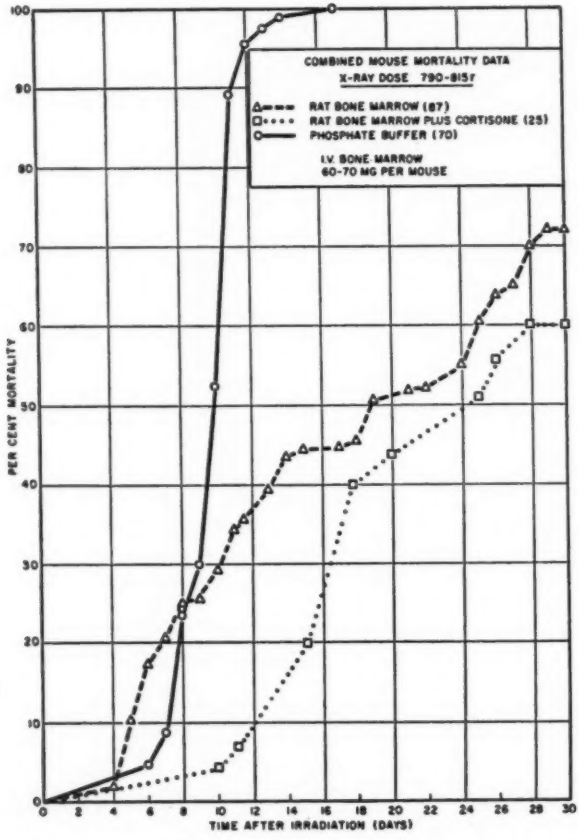
*Nonsurvivors at 30 days.
†Marrow injected 24 hours after exposure.


BODY WEIGHT CHANGE (PER CENT)
TIME AFTER IRRADIATION (DAYS)
X-RAY DOSE 800r
CONTROL - SUCROSE BUFFER (15)
RAT BONE MARROW (16)
RAT BONE MARROW PLUS CORTISONE (7)
(CORTISONE PRE AND POST)
I.V. BONE MARROW
70 MG PER MOUSE

TEXT-FIGURE 1.—Comparative body-weight changes in X-irradiated mice (800 r) injected with rat bone marrow, with rat bone marrow plus cortisone, and with sucrose buffer.

Histologic examination of fixed and stained femur sections revealed a striking difference in the appearance of the bone marrow in the marrow-treated mice, as compared with that of the control mice. The sections from marrow-injected mice showed large areas of megakaryocytes, foci of erythropoiesis, apparent reticuloendothelial hyperplasia, and numerous collections of mature polymorphonuclear leukocytes—a very active marrow in all respects. By contrast, the marrow from the control mice was almost completely acellular and atrophied; hemorrhage and pigmentation were visible.

The combined mouse data (involving approximately 150 mice) showing the time-course of mortality for irradiated mice receiving rat bone marrow *versus* phosphate buffer are summarized in text-figure 2. Two points of interest are indicated by these data: 1) About 30 percent of the bone-marrow-treated mice were alive 30 days after irradiation, whereas none of the phosphate buffer group survived. 2) Approximately one third of



TEXT-FIGURE 2.—Effect of rat bone-marrow injection and of rat bone marrow plus cortisone on mortality of mice after lethal total-body X irradiation.

the marrow-treated mice died relatively late, that is between the 14th and 30th day postexposure. This time sequence of deaths contrasts with that seen among X-irradiated LAF_1 mice injected with homologous (*e.g.*, LAF_1) mouse bone marrow, in which case very few, if any, deaths occur after the second week (*5*); the sequence of deaths also contrasts with that seen among irradiated Sprague-Dawley rats treated with homologous rat bone marrow (*8*).

Effect of Rat Bone Marrow Plus Cortisone

It is well known that cortisone administration to animals elicits a depression in the immune response to antigenic substances. Toolan (*11*) has succeeded in growing human tumor tissue in cortisone-treated laboratory animals. In view of these results it was considered possible that the injected marrow might better be able to "take" in a heterologous host animal treated with cortisone. Several different schedules of cortisone administration (1.25 mg. per injection) were tried, as follows: *Experiment 1:* One injection per mouse on the day before irradiation, one on the day of irradiation (2 hours after exposure), and one on the 4th day postirradiation, designated −1, 0, 4; *Experiment 2:* −1, 0, 3, 13, 24, 26; *Experiment 3:* −1, 0, 1; *Experiment 4:* 1, 3. The results of these experiments (table 2) suggest that cortisone administration may under certain conditions enhance the postprotective effect of rat bone marrow on irradiated mice. The time plot of the mortality data (text-fig. 2) indicates a prolonged survival time among the mice treated with marrow plus cortisone, as compared with those injected with marrow only.

Surviving animals were observed beyond the 30-day period, in view of the late deaths which occurred up to the end of the first month. At 45 days 9.5 percent of the mice treated with marrow were alive; at 60 days 6.7 percent. Among the irradiated mice treated with bone marrow and cortisone, 19 percent survived at 45 days and 9.7 percent at 60 days.

Postirradiation body-weight changes among the three groups of irradiated mice in Experiment 1 (table 2) are shown in text-figure 1.

TABLE 2.—*Effect of rat bone marrow plus cortisone on survival of X-irradiated mice*

Expt. No.	X-ray dose (*r*)	Treatment	30-Day survival		Mean survival time* (*days*)
			No./total	Percent	
1	800	Rat bone marrow (70 mg.)	4/16	25	16.3
	800	Rat bone marrow (70 mg.) + cortisone (−1, 0, 4)	4/7	57	13.8
	800	Sucrose buffer	0/15	0	9.5
2	815	Rat bone marrow (66–70 mg.)	2/12	17	15.0
	815	Rat bone marrow (66–70 mg.) + cortisone (−1, 0, 3, 13, 24, 26)	4/9	44	18.8
	815	Sucrose buffer	0/10	0	9.3
3	800	Rat bone marrow (70 mg.)	1/5	20	21.5
	800	Rat bone marrow (70 mg.) + cortisone (−1, 0, 1)	3/6	50	15.5
	800	Sucrose buffer	0/11	0	9.1
4	800	Rat bone marrow (70 mg.)	1/6†	17	16.5
	800	Rat bone marrow (70 mg.) + cortisone (1, 3)	5/14‡	36	18.5
	800	Sucrose buffer	0/4	0	10.1
	800	Sucrose buffer + cortisone (1, 3)	0/4	0	10.2

*Nonsurvivors at 30 days.

†This group originally comprised 10 mice; 2 were sacrificed on day 12 and 2 on day 18.

‡This group originally comprised 18 mice; 2 were sacrificed on day 18 and 2 on day 24.

Deoxyribonucleic Acid Content in Spleen

Recent studies from this laboratory (*10*) have shown that the deoxyribonucleic acid (DNA) level in the spleen provides a chemical index of recovery and survival from acute injury in X-irradiated mice treated with spleen homogenate. This criterion of recovery has been employed in the present experiments. Table 3 summarizes the observed analytical values for spleen DNA content in individual animals taken from groups of irradiated mice treated with rat bone marrow, or with marrow plus cortisone. The hematopoietic regeneration elicited by the marrow treatment is reflected in these values. By the third week post-treatment the spleen DNA levels in the marrow-injected mice had come up to concentrations approaching that of normal mice; and the total spleen DNA content in some instances exceeded that of normal mice. As reported previously (*10*), the total spleen DNA content of normal nonirradiated female LAF_1 mice (14–16 weeks old) was 1.96 mg. ± 0.07; the spleen DNA concentration, 19.7 ± 0.35 μg. per mg. The following DNA values for the spleen were observed in mice 9 days after exposure to lethal, total-body X irradiation (740 r) and injected with phosphate buffer: total DNA content per spleen, 244 ± 16 μg.; DNA concentration, 6.43 ± 0.60 μg. per mg.

TABLE 3.—*Effect of rat bone-marrow injection on spleen deoxyribonucleic acid content of X-irradiated mice (800 r)*

Treatment	Time after X ray (*days*)	Spleen DNA content	
		Total per spleen (*mg.*)	Concentration (*μg. per mg.*)
Rat bone marrow	9	1. 31	11. 0
Rat bone marrow + cortisone	9	1. 77	17. 6
Buffer	9	0. 19	3. 6
Rat bone marrow	12	2. 13	12. 6
Rat bone marrow + cortisone	12	2. 79	15. 6
Rat bone marrow	12	2. 10	14. 3
Rat bone marrow	12	3. 68	14. 3
Rat bone marrow	12	2. 39	13. 5
Rat bone marrow	12	1. 75	13. 8
Rat bone marrow	12	1. 65	13. 1
Buffer	12	0. 57	12. 1
Rat bone marrow	15	0. 77	9. 55
Rat bone marrow	15	1. 25	13. 5
Rat bone marrow	15	2. 07	12. 5
Rat bone marrow	18	3. 0	16. 5
Rat bone marrow	18	2. 35	10. 5
Rat bone marrow + cortisone	18	3. 75	12. 2
Rat bone marrow + cortisone	18	1. 40	10. 6
Rat bone marrow + cortisone	25	2. 0	12. 8
Rat bone marrow + cortisone	25	5. 37	14. 1

Discussion

The present results confirm those of Congdon and Lorenz (*7*) showing the feasibility of postprotection of X-irradiated mice (at the LD_{100} dose level) by a single intravenous injection of rat bone-marrow suspension. They show, furthermore, that a significant number of the treated mice were alive 30 days after exposure, and as late as 60 days.

As an additional criterion of recovery, the data on DNA content in the spleen show that the injection of rat bone marrow into the irradiated mice elicited hematopoietic regeneration by the end of the second week. Similarly the upturn in blood leukocyte values is indicative of bone-marrow regeneration. It is not known at present whether these manifestations of hematopoietic regeneration are the cause or result of recovery processes occurring in the treated animal. However, it is perhaps not premature to inquire whether the regenerating bone marrow and spleen in the mice injected with rat marrow are of rat or of mouse origin. Three distinct formulations of this question can be stated:

1) Is hematopoietic recovery the resultant of repopulation of the mouse marrow by the direct progeny of the injected rat bone-marrow cells? (A positive answer would imply that the regenerating marrow in the mouse actually consists of rat-marrow cells.)

2) Is the regeneration of mouse marrow a spontaneous, autochthonous recovery process made possible by "tiding over" of the animal by the functionally intact rat marrow?

3) Does the injected rat marrow "take" and produce a "humoral" (*e.g.*, subcellular or acellular) principle which evokes or stimulates regeneration of the host marrow?

Congdon and Lorenz (*7*) consider it "highly improbable that the rat bone-marrow cells repopulate the bone marrow of the mouse or transform into cells that repopulate the lymph nodes, splenic nodules or thymus of the mouse." According to these authors, intraperitoneally injected rat bone marrow survives in the peritoneal cavity of the mouse, and is therefore potentially able to produce a humoral protective factor which may itself not be species-specific. If this explanation were correct, one would expect to see a similar pattern of protection with heterologous as with homologous bone-marrow treatment. However, the present experimental data, as well as those of Congdon and Lorenz, indicate that a relatively large percentage of late deaths (*e.g.*, after 2 weeks) occurs in the irradiated mice injected with rat marrow; in contrast, relatively few late deaths have been observed among irradiated mice (*5*) and rats (*8*) treated with homologous bone marrow (radiation dose level 750 to 900 r). The relatively large percentage of late deaths occurring in the present series of experiments is difficult to interpret in view of the definite signs of hematopoietic recovery evident by the end of the second week.[5] At-

[5] Late deaths occurring during this same time period were observed in homologous bone-marrow-treated rats exposed to X-ray dose levels between 1,200 r and 1,400 r, with the intestine shielded during exposure (*18*). A significant number of late deaths (*e.g.*, 15 to 30 days) have been observed in a large series of LAF_1 mice receiving total-body X irradiation in the lethal range (*13*).

tempts are now being made to investigate this question in terms of possible delayed immunologic response of the host to the heterologous marrow. The studies of Barnes and Loutit (*14*) are of interest in this connection.

The experimental data presented here suggest that cortisone treatment of X-irradiated mice may enhance postirradiation protection by heterologous bone marrow. Although the enhancement is relatively slight, it is hoped that the use of cortisone and/or similar modifying agents will provide experimental tools for further investigations of the phenomenon of radiation postprotection by heterologous bone marrow—one of the experimental areas in radiobiology in which Egon Lorenz was the pioneer.

Summary

Rat bone-marrow suspension was injected intravenously into mice following their exposure to lethal total-body X irradiation (800 r). Up to 50 percent of mice thus treated were alive after 30 days, whereas none of the control mice, injected with buffer, survived. Regeneration of the hematopoietic system, elicited by the heterologous marrow injection, was evidenced by the recovery of blood leukocyte count, by the histologic appearance of the bone marrow, and by the return of spleen DNA content to near-normal values.

A relatively large percentage of late deaths, *i.e.*, between 2 weeks and 2 months after irradiation, was observed among the mice treated with rat bone marrow.

In some experiments the irradiated mice received injections of cortisone in conjunction with the bone-marrow treatment. The data suggest that the administration of cortisone may enhance the protective effect of rat bone marrow in X-irradiated mice.

The possible mechanisms of radiation protection by heterologous bone marrow have been discussed.

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A Comparison of the Patterns of Excretion and the Carcinogenicity in the Rat of 2-Acetylaminofluorene and Related Compounds[1,2]

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The excretory products of a chemical carcinogen may provide information on the absorbability and detoxication of the substance without contributing to a knowledge of the carcinogenic mechanism involved. However, the excretion of more than 90 percent of the isotopic nitrogen of 2-acetylaminofluorene-N^{15} (2-AAF-N^{15}) (*1*) and the radioactivity of 2-AAF-9-C^{14} (*2*) by the rat within 4 to 6 days following oral administration led us to hope that identification of the products of excretion of this carcinogen might throw new light on the mechanism of carcinogenesis.

Two thirds to three fourths of a single dose of 2-AAF-N^{15} are recoverable as isotopic nitrogen from the urine of the rat and the remainder from the feces. The diazotization-R-salt-coupling test for 2-AAF, corrected for the lesser color density, at 525 mμ, of the metabolite 7-hydroxy-2-acetylaminofluorene (7-OH-2-AAF) (*3*), rarely accounts for as much as 80 percent of the excreted N^{15}. This is due in part to inability to extract quantitatively the material from the feces, but the urine also contains more N^{15} estimated material than can be accounted for colorimetrically (*1*, *3*). The material excreted in the urine following 2-AAF administration consists of at least six compounds; namely, unmetabolized 2-AAF, 2-AF (*4*, *5*), 7-OH-2-AAF, 7-OH-2-AF (*4–6*), 1-OH-2-AAF, and 3-OH-2-AAF (*7*). These last two metabolites accounted for only 1.5 and 3 percent, respectively, of the administered dose of 2-AAF. They do not interfere significantly in the nitrite test for 7-OH-2-AAF nor do they give any interfering color in the diazotization-R-salt-coupling test (*3*, *7*).[5]

7-OH-2-AAF or metabolites that react similarly in the nitrite test (*3*) account for approximately 44 to 58 percent of the total diazotizable

[1] Received for publication November 22, 1954.

[2] Presented in part at the meeting of the American Association for Cancer Research at Chicago, April 1953.

[3] Acknowledgment is made of the indispensable technical assistance of Clara Fischer Smith.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[5] The Weisburgers (abstract, Biological Chemistry Division, A. C. S., 127th annual meeting, March, 1955) have now demonstrated the presence of 5-hydroxy-2-AAF (5-OH-2-AAF) in the urine of rats following administration of 2-AAF-9-C^{14}. Since they found that 5-OH-2-AF interferes in the nitrite test for 7-OH-2-AF at 450 mμ to the extent of 36 percent and with 2-AF in the diazo-R-salt-coupling test at 525 mμ to 65 percent, it must be concluded that the diazo-R-salt test on the excreta, after 2-AAF, and possibly after other 2-amino derivatives of fluorene, accounts for the 5-hydroxy- as well as the 7-hydroxy-derivative in addition to 2-AF and 2-AAF, and that the nitrite test measures the 5-hydroxy-metabolite in addition to 7-hydroxy-2-AAF.

R-salt-coupling material in the urine of the rat. 7-OH-2-AAF therefore appears to be a major metabolite of 2-AAF. Some free 7-OH-2-AAF is present in the urine, but most of it is conjugated as a glycuronide and a small amount may exist as a sulfate (*4*, *5*).

This report compares the patterns of excretion of some derivatives of 2-AF and of several structurally related substances with respect to the excretion of total diazotizable material and of diazotizable metabolites that react in the test for 7-OH-2-AAF, to see whether any relation exists between the excretory behavior of the compounds and their ability to induce tumors. The compounds studied have been tested for carcinogenicity by Morris[6] of this laboratory or by other investigators.

Materials and Procedure

Thirty-five young adult male Buffalo rats were maintained in glass metabolism cages designed to separate the urine and feces and fed *ad libitum* a diet of natural foodstuffs, Morris diet no. 222 (*8*). Single doses of 10 mg. of 2-AAF in propylene glycol (or the molecular equivalent of related substances) per 100 grams of body weight were administered to 31 of the rats—to 29 by stomach tube and 2 by intraperitoneal injection. Four rats, one with each of four compounds, received double the dosage by stomach tube. Five compounds were administered each to 1 rat, each of eleven to 2 rats, one to 3, and one compound to 5 rats. The urine was collected in iced tubes during successive 7- or 8- and 16-hour periods from the time of administration of a compound until diazotizable urinary material was no longer detectable (4–8 days).

The urine and acetone extracts of the feces were analyzed by the diazotization-R-salt-coupling (diazo) test for 2-AAF (*9*) and by the spectrophotometric method (nitrite test) for 7-OH-2-AAF (*3*). The urine was usually analyzed before and after hydrolysis to distinguish the free and conjugated amines. The values for total diazotizable material in the diazo test were obtained by correcting the values for 7-OH-2-AAF determined by the nitrite test (*3*). Isotopic nitrogen was also used as a tracer with 2-AF, 2-AAF, and 2-nitrofluorene (2-NF). The urine was analyzed, after suitable dilution, without filtering. Diazotizable material was removed from the feces by repeated acetone extraction. The acetone was evaporated at room temperature and the dry residues were taken up in measured volumes of glacial acetic acid. The acetic acid solutions were used for the colorimetric analyses. Isotopic nitrogen was determined on samples of dry ground feces and rarely on acetone extracts.

The compounds studied were synthesized by Drs. Elizabeth and John Weisburger, of this laboratory, and by Dr. Francis E. Ray and his associates.[7]

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[6] Sincere appreciation is expressed to Dr. Harold P. Morris for furnishing all the available data in his studies of carcinogenicity of the compounds.

[7] To the Weisburgers, grateful acknowledgment is made of samples of the following compounds: 7-OH-2-AAF; 2-di-AAF; 2,7-di-AAF; 2-2′-diacetylamino-9,9′-bifluoryl; 2,2′-diacetylamino-9,9′-bifluorylidene; 3-iodo-2-AAF, 7-iodo-2-AAF; 3-AAF; 4-AAF; 2-OH-3-AAF and 1-OH-4-AAF; and to Dr. Ray of the Cancer Research Laboratory, University of Florida, Gainesville, Fla., for preparation of: 2-AF-N^{15}, 2-AAF-N^{15}; 2-NF-N^{15}; 2-monomethyl-AF; 2-dimethyl-AF; 2-BAF; 4-acetylaminobiphenyl; and 4,4′-diacetylbenzidine.

Compounds making up group 1, with the exception of 7-OH-2-AAF and 2,7-diacetylaminofluorene (2,7-di-AAF), consisted of derivatives of 2-AF that might yield 2-AF and/or 2-AAF on metabolism. These derivatives were 2-AF, 2-AAF, 2-NF, 2-diacetylaminofluorene (2-di-AAF), 2-monomethyl-AF, 2-dimethyl-AF, 7-OH-2-AAF, 2,7-di-AAF, 2-benzoyl-AF (2-BAF), 2-2′-diacetylamino-9,9′-bifluoryl, and 2,2′-diacetylamino-9,9′-bifluorylidene.

7-OH-2-AAF and 2,7-di-AAF are the only substances in group 1 that give, before metabolism by the animal, a significant color in the nitrite test for 7-OH-2-AAF, 2-7-di-AAF yielding approximately one fifth the color of the chromogen from 7-OH-2-AAF (*3*). Four of the substituted compounds give quantitatively the expected color in the diazo test for 2-AF assuming that the substituent groups in the amino groups are lost quantitatively by hydrolysis in the analytical procedure.[8] Those compounds that do not react like 2-AF after acid hydrolysis are the monomethyl and dimethyl derivatives of 2-AF, which are not significantly demethylated (*3*); the nitro, which is not reduced by the analytical procedure; the benzoyl, which is only partially hydrolyzed; and 7-OH-2-AAF, which produces 50 percent of the color density when coupled with R-salt of an equal weight of 2-AAF.

The materials administered and the R-salt-coupling materials recovered were estimated as millimolecular equivalents of 2-AAF. The nitrite-reacting metabolites recovered were estimated as millimoles of 7-OH-2-AAF.

A second group of substances, related structurally to 2-AAF, but not expected to be metabolized to 2-AF or 2-AAF, was investigated. These compounds of group 2 are fluorene, 3-AAF, 4-AAF, 3-iodo-2-AAF, 7-iodo-2-AAF, 4-acetylaminobiphenyl, and 4,4′-diacetylbenzidine. Except for fluorene they are diazotizable. The diazonium derivatives couple with R-salt as in the method used for the quantitative estimation of 2-AF and 2-AAF. As the color densities of the chromogens so formed are different from those of 2-AAF, the recoveries of R-salt-coupling material were estimated by comparison with standard curves of the administered compounds.

The substituent iodine groups of the 3- and 7-iodo derivatives of 2-AAF appear somwhat unstable in acid medium and thus influence the spectrophotometric reactions. During the addition of nitrite for diazotization a yellow color develops having a color density at 450 mμ, varying with dilution, from 17 to 23 percent of that of the nitrite-produced chromogen from 7-OH-2-AAF. 3-AAF, 4-AAF, 4-acetylaminobiphenyl, and 4,4′-diacetylbenzidine do not interfere in the spectrophotometric test for 7-OH-2-AAF, giving negative reactions before metabolism by the rat. The nitrite-reacting material which appeared after 4-acetylaminobiphenyl was estimated for convenience as 7-OH-2-AAF. No correction could be made for any probable difference in the color density of the unknown material

[8] The color follows Beer's Law in a limited range of concentrations. Samples were diluted to approach these optimum ranges.

and 7-OH-2-AAF, since no possible metabolites were available for investigating their behavior in the colorimetric tests.

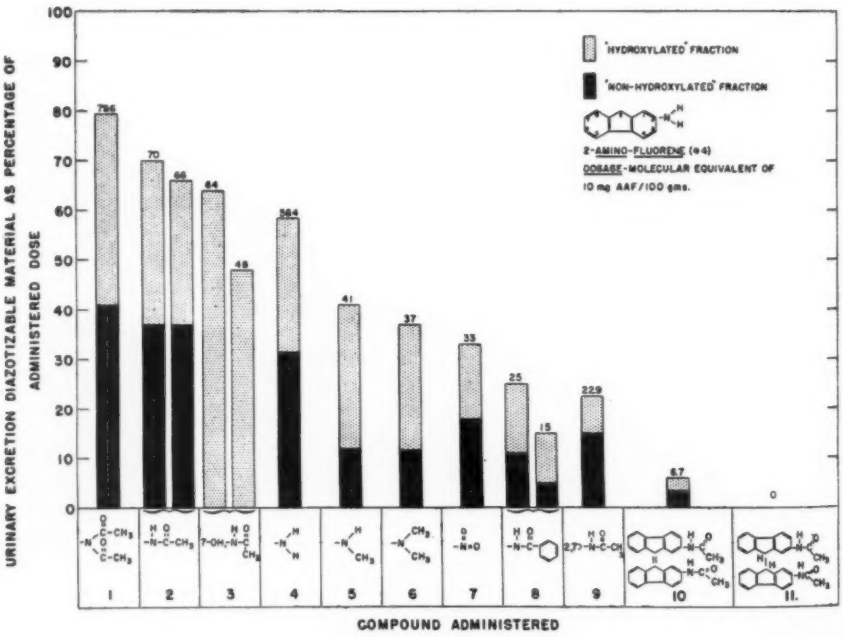
The nitrite-reacting material found in the urine following administration of 3-AAF and 4-AAF was estimated as 2-OH-3-AAF (*10*) and 1-OH-4-AAF, respectively. High concentrations of these compounds give green colors in the presence of the ammoniacal solution of R-salt in the diazo test, but the low urinary concentrations did not interfere in the diazo method of analysis. 2-OH-3-AAF gives a pinkish-yellow color in the nitrite test which has a color density 15 percent of that given by 7-OH-2-AAF, while 1-OH-4-AAF gives a typical yellow color and a color density 10 to 17 percent that of 7-OH-2-AAF, the percent difference in density decreasing with increased dilution.

Diacetylbenzidine and the bifluoryl compound were administered intraperitoneally in propylene glycol, each to one rat.

Results

Text-figures 1 and 2 summarize graphically recoveries from the urine and feces of total diazotizable and nitrite-reacting material for the compounds in group 1.

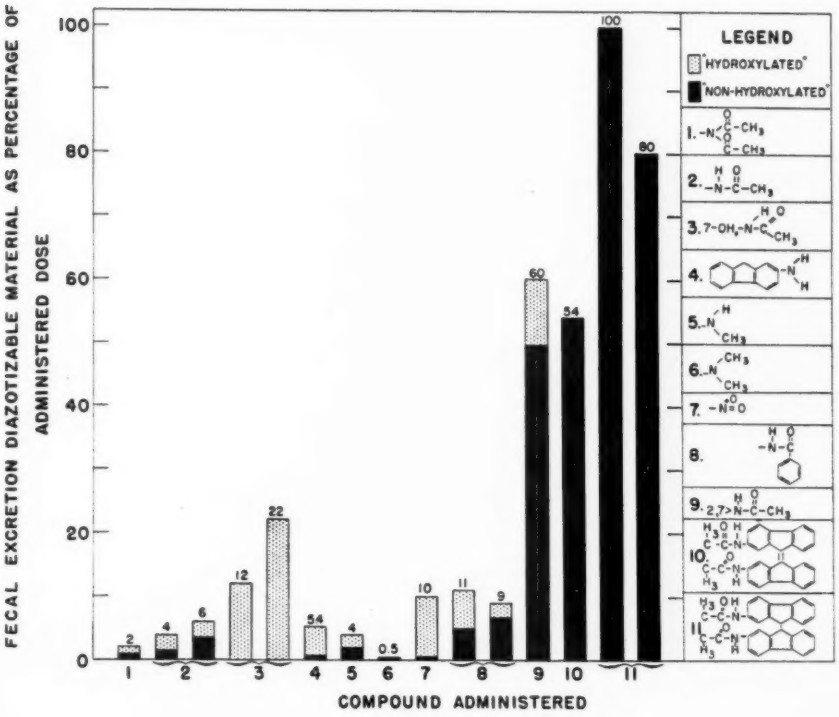
Recoveries from rat urine of 31 to 36 percent of administered 2-AF (compound 2, 2a, table 1) as nonhydroxylated diazotizable material are much larger than the expected urinary excretion of 2-AF and 2-AAF based on carrier isotope dilution methods (*4*, *5*).



TEXT-FIGURE 1.

The data in text-figures 1 and 2 and in the last two columns of table 1 indicate that 2-di-AAF (compound no. 1), 2-AAF (nos. 2 and 2a), 2-AF-2-methyl-AF, 2-dimethyl-AF, 2-NF, 2-BAF (nos. 4 through 8), and 2,2′, diacetylamino-9,9′-bifluorylidene (no. 10) were metabolized by the rat to some extent as evidenced by the appearance in the urine and feces of material that reacts in the nitrite test like 7-OH-2-AAF. These compounds were negative in this test before passage through the animal.

The recoveries of total corrected diazotizable material from the urine and feces after administration of compounds of group 1 are always less than the amount of diazotizable or potentially diazotizable material



TEXT-FIGURE 2.

administered. It is likely that other nondiazotizable R-salt-coupling metabolites of 2-AAF and 2-AF, in addition to 1- and 3-OH-2-AAF, or metabolites that yield chromogens of lesser color densities than 7-OH-2-AAF in both the coupling and the nitrite tests, may also be excreted in the urine. Other 2-amino derivatives of fluorene that are metabolized by the rat may also lead to the excretion of metabolites that are not estimated quantitatively as 2-AF and 7-OH-2-AAF.

It is reasonable to assume that appearance of positive diazotizable R-salt and nitrite reactions in the excreta indicates the reduction of NF

TABLE 1.—*Recovery of diazotizable material from excreta of rats**

Compound administered	Diazotization-R-salt-coupling material				Nitrite-reacting material†	
	Urine		Feces		Urine	Feces
	Percent of administered		Percent of administered		Percent of total diazotizable material recovered from urine	Percent of total diazotizable material recovered from feces
	Uncorrected	Corrected for 7-OH-2-AAF†	Uncorrected	Corrected for 7-OH-2-AAF†		
	Group 1					
1) 2-Diacetylaminofluorene	60;40	80;51	1;4	2;5	48;39	60;44
2) 2-Acetylaminofluorene	53;47(49‡)	70;66(67‡)	3;5(8‡)	4;6(16‡)	47;44(56‡)	62;42(98‡)
2a) 2-Acetylaminofluorene following repeated 2-AAF	50;51	67;69	.....	.....	52;58	.....
3) 7-Hydroxy-2-acetylaminofluorene	0;0	64;48	0;0	12;22	100;100	100;100
4) 2-Aminofluorene	44(26‡)	58(38‡)	3(4‡)	5(7‡)	44(57‡)	88(70‡)
5) 2-Monomethylaminofluorene	27(26‡)	41(36‡)	3(2‡)	4(2‡)	72(52‡)	50(66‡)
6) 2-Dimethylaminofluorene	25(3‡)	37(5‡)	0.5(0.7‡)	0.5(0.7‡)	68(76‡)	0(0‡)
7) 2-Nitrofluorene	26;21	33;25	5;4	10;5	45;32	95;21
8) 2-Benzoylaminofluorene	18;10	25;15	8;7	11;9	56;66	54;25
9) 2,7-Diacetylaminofluorene	19;26	23;30§‖	55;50	60;56§‖	32;29§‖	16;23§
10) 2,2′-Diacetylamino-9,9′-bifluorylidene	5	7	54	54	49	0
11) 2,2′-Diacetylamino-9,9′bifluoryl	0;0(0¶)	0;0(0¶)	100;80(0¶)	100;80(0¶)	0;0(0¶)	0;0(0¶)
	*Group 2***					
12) Fluorene	0	0	0	0	0 †	0
13) 3-Acetylaminofluorene	43	43	12	12	60 ††	0
14) 4-Acetylaminofluorene	49	49	5	5	189 ††	0

15)	3-Iodo-2-acetylaminofluorene....	18;5	.....§	38;12	.....§	292;279**	115;151**
16)	7-Iodo-2-acetylaminofluorene....	27(22‡‡)	.....§	30(1‡‡)	.....§	160**(300**‡‡)	107**(640**‡‡)
17)	4-Acetylaminobiphenyl.....	28	.....	3	3	37‖	0
18)	4,4′-Diacetylbenzidine.....	0(0¶)	0(0¶)	55(0¶)	55(0¶)	0(0¶)	0(0¶)

*In the first column of data each figure, separated by semicolons (or parentheses, where footnote refers to one rat of group), represents the recovery from one rat. The data for the same rat follows through horizontally in all of the columns. Recoveries were estimated as milliequivalents of 2-AAF and of 7-OH-2-AAF unless otherwise indicated.

†The nitrite test was developed for the determination of 7-OH-2-AAF. This hydroxylated derivative gives 50 percent of the color density of 2-AAF in the diazotization-R-salt-coupling reaction.

‡Dosage for animals with this footnote was twice that of the molecular equivalent dose of 10 mg. (0.045 millimoles) of 2-AAF per 100 gm. body weight which was administered to all other rats.

§2,7-Diacetyl-AF, before administration, interferes with the nitrite test to the extent of 20 percent and the 3- and 7-iodo-derivatives of 2-AAF interfere up to 20 and 23 percent, respectively. The recovered nitrite-reacting material from these compounds would not represent 7-OH-2-AAF and may not indicate metabolized derivatives but interference due to the substituent group in the 7-position of compound 9 and to instability of the iodine substituents in acid medium for compounds 15 and 16.

‖Estimated incorrectly as 7-OH-2-AAF, since no hydroxy derivatives or other possible nitrite-reacting metabolites of these compounds are available for comparison.

¶Administered intraperitoneally to one rat.

**Recoveries for group 2 were estimated from standard curves made for diazotization-R-salt-coupling and for nitrite tests of the material administered except where otherwise indicated. Fluorene is nondiazotizable before and after passage through the rat.

††The nitrite-reacting material recovered after 3-AAF was calculated as 2-OH-3-AAF and after 4-AAF as 1-OH-4-AAF. Neither of these hydroxy derivatives interfered in the diazotization-R-salt-coupling test.

‡‡Two days after administration of the compound, this rat, #100, appeared to have middle-ear infection. Little, if any, food was eaten.

and the demethylation of the two methyl derivatives, at least to the extent indicated by the analyses.

The failure of 2-BAF to react in the nitrite test and the 50-percent lower color density of the R-salt chromogen of diazotized 2-BAF compared to that of 2-AAF makes impossible the quantitative estimation of nitrite-reacting and nitrite-nonreacting fractions from 2-BAF.

The only evidence for the metabolism of 7-OH-2-AAF is the failure to account for more than 76 percent of that administered.

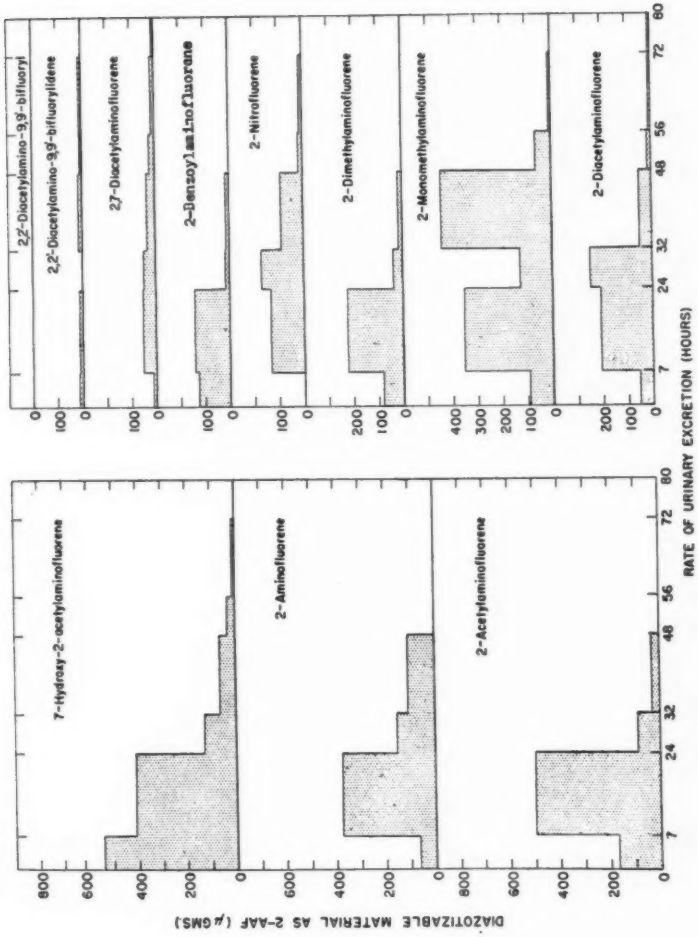
Eleven percent, or less, of the potentially diazotizable administered material of 2-di-AAF, 2-AAF, 2-AF, 2-monomethyl- and 2-dimethyl-AF, 2-NF, and 2-BAF (compounds nos. 1, 2, and 4–8) was detectable in the acetone extracts of the feces. The bifluoryl compound (no. 11), when administered by gastric intubation, was detected only in the excreta from the large intestine. It is possible that the recovery of less than 100 percent of the administered dose in one rat was due to failure to extract the material from the feces quantitatively.

More than half of the bifluorylidene compound (no. 10) administered was excreted in the feces with no evidence of metabolism. Of the small amount of diazotizable material excreted in the urine, 49 percent behaved like 7-OH-2-AAF.

The compounds in group 1, table 1, fall roughly into three subgroups with regard to the total amounts of diazotizable and metabolized diazotizable material excreted in the urine: 2-di-AAF and 2-AAF (nos. 1, 2, 2a), and possibly 2-AF (no. 4), showing the highest yields; 2-methyl-, 2-dimethyl-AF, 2-NF, and 2-BAF (nos. 5–8), yielding somewhat lesser amounts; and the bifluorylidene compound (no. 10) yielding considerably less. Compound no. 3, 7-OH-2-AAF, falls into the first subgroup and 2,7-di-AAF (no. 9), falls into the second subgroup. However, there is no real proof of the metabolism of 7-OH-2-AAF and the evidence for the metabolism of 2,7-di-AAF is questionable since the compound administered shows 20-percent interference in the nitrite test, and there is no information available on the spectrophotometric behavior of any metabolite of the 2,7-derivative.

Some patterns of urinary excretion of the materials are presented in text-figure 3. The average amounts of total diazotizable material found in the urine during the 7- and 16-hour periods are presented for one representative rat for each compound of group 1. The only differences observed in the patterns of excretion were an earlier appearance of the peak of excretion of diazotizable material in the urine following administration of 7-OH-2-AAF, considerable variation in the average amounts excreted per period after the different compounds, and significantly smaller amounts excreted after the less potent carcinogens.

The recoveries calculated from diazotizable and isotopic nitrogen after 2-AAF, 2-AF, and 2-NF are presented in table 2. The recovery of diazotizable material in the urine, corrected for 7-OH-2-AAF, never quite equals that of the recovery of N^{15}. With one exception, the isotopic nitrogen recovered in the feces is much greater than that of diazotizable



TEXT-FIGURE 3.

nitrogen. It is questionable whether the isotopic nitrogen that is difficult to extract from the feces is in a diazotizable form, and the recovery of twice as much N^{15} as diazotizable nitrogen from acetone extracts of three of the rats suggests that a large portion of the acetone-extractable isotopic nitrogen of the feces is nondiazotizable.

TABLE 2.—*Comparison of recoveries of diazotizable material and of isotopic nitrogen following gastric administration of fluorene derivatives to the rat*

Compound administered*	Recoveries† as percent of administered dose					
	Urine			Feces		
	Diazotizable			Diazotizable‡		
	Uncorrected for "7-OH-2-AAF"	Corrected	N^{15}	Uncorrected for "7-OH-2-AAF"	Corrected	N^{15}
2-Acetylaminofluorene-N^{15}	53	70	81	3	4	22
	47	66	75	5	6	23
	49§	67§	79§	8§	16§	15§
2-Aminofluorene-N^{15}	44	58	69	3	5	24
	26§	38§	68§	4§	7§	22§
2-Nitrofluorene-N^{15}	26	33	68	5	10	36
	21	25	68	4	5	30

*Materials were administered in propylene glycol in doses of 10 mg. 2-AAF/100 gm. body weight, or the molar equivalent, except where otherwise indicated.

†Recoveries, calculated as millimoles of 2-AAF, were compared with millimoles of the compounds administered.

‡The diazotizable material of the feces was recovered from acetone extracts of the feces whereas ground feces were used for determination of isotopic nitrogen.

§Dosage was 20 mg. 2-AAF/100 gm. body weight or the molecular equivalent.

More experimental data are needed to determine whether there is any significance to the lower recoveries of diazotizable and of isotopic nitrogen from the urine of the rats that received 2-AF compared with the recoveries after 2-AAF. There was less diazotizable material in the urine of the rats that received 2-NF than after 2-AF, but the percentage of the administered N^{15} in the urine was the same. The fraction of administered N^{15} recovered from the feces appears to be greater after 2-NF than after 2-AF and 2-AAF.

Among the compounds in group 2, fluorene is not diazotizable and was therefore not detectable by the spectrophotometric methods used.

The total recoveries of diazotizable material from 3-AAF and 4–AAF (nos. 13 and 14, table 1) were 55 and 54 percent, respectively, of the amounts administered. The nitrite-reacting fraction from the urine of the rat administered 3-AAF, as mentioned previously, was estimated as 2-OH-3-AAF. Like the synthetic compound the material in the urine gave a pinkish-yellow color in the nitrite test. The amount of 2-OH-3-AAF estimated for the urine was 60 percent of the diazotizable material found to be present as 3-AAF by the R-salt-coupling method of analysis. Since no interfering green color was observed in the diazo method from the 2-OH-3-AAF present, the total recovery of material coming from the administered 3-AAF would be the sum of the recoveries by the two methods of analysis from urine and feces, or 81 percent of the dose administered.

The urine of the rat that received 4-AAF yielded a typical yellow color in the nitrite test. It was estimated as 1-OH-4-AAF, the only hydroxy derivative of 4-AAF that was available for comparison. The amount estimated as 1-OH–4–AAF was found to equal 189 percent of the detectable diazotizable material. Since no interference of green color with the diazo test was observed, if 1-OH-4-AAF were the only metabolite responsible for the nitrite test, the sum of the recoveries by the two methods should represent the total recovery of material from the administered 4-AAF. This amounts to 147 percent of the dose administered to the rat.

4-Acetylaminobiphenyl, compound no. 17, table 1, was transformed in part to a metabolite(s) which gave a positive reaction for 7-OH-2-AAF. The diazotizable material was present chiefly in the urine, but no correction was made for possible differences in the spectrophotometric reactions of metabolites and the material administered.

The ratio of nitrite-reacting to total diazotizable material in the urine, calculated from standard curves of the iodo derivatives in these two reactions, is approximately 3 for both rats after 3-iodo-2-AAF (no. 15, table 1) and 1.6 and 3, respectively, for the two rats given the 7-iodo derivative (no. 16), indicating that the compounds have been metabolized to products that yield more color in the nitrite test than the compounds administered. The appearance in acetone extracts of the feces of 1.1 and 1.5 times as much nitrite-reacting material as would have been expected (100 percent) from the unmetabolized 3-iodo-2-AAF may indicate the presence of a small amount of metabolite, but the great differences observed in the fecal excretion of the two rats administered 7-iodo-2-AAF cannot be explained unless the results obtained with the second rat were affected by a condition that developed on the second day, the signs of which suggest middle-ear infection. This rat ate little, if any, food and excreted little feces (see table 1‡‡ and table 3¶).

4,4′-Diacetylbenzidine, no. 18, table 1, was not detectable in the urine but 55 percent of the amount administered was recovered in the feces. The rat was killed one week after administration of the compound. Seventy-five percent of the material recovered was from two samples of feces collected during the first 48 hours. Inability to extract completely the material from the feces may account in part for the failure to recover quantitatively 4,4′-diacetylbenzidine because the final re-extraction of the combined extracted samples contained 15 percent as much diazotizable material as that recovered during the first three acetone extractions of the individual samples.

No diazotizable material was detected in the urine or feces of the two animals that received the bifluoryl derivative and the 4,4′-diacetylbenzidine by parenteral administration. Two weeks after dosage the rats were sacrificed. Fused intimately into the surfaces of the liver and spleen were white nodules. Sections were made of tissues containing these nodules, while some of the nodular material was extracted with acetone, and after evaporation of the solvent, the residues were analyzed for diazotizable and nitrite-reacting material. Strong positive tests in the R-salt-coupling

TABLE 3.—*Comparison of urinary excretion of diazotizable material by rats following gastric administration of fluorene derivatives and related compounds**

Compound	No. of rat	Percent of total recovery	Average rate of excretion (*μg./hour*)	Conjugated amino groups as percentage of total†	Average daily change in body weight (*gm.*)
		Group 1‡			
2-Diacetylaminofluorene	59	97	82	93	+1.6
	60	91	87	—	—
2-Acetylaminofluorene	46	94	224	94	+0.7
	54	92	195	94	−3.0
	39A	81§	333	92	−3.0
7-Hydroxy-2-acetylaminofluorene	47	84	219	100	−0.7
	53	68	202	100	+0.3
2-Aminofluorene	45	91	197	90	−2.3
	38A	85§	210	80	−3.4
2-Methylaminofluorene	55	91	180	94	+2.2
	38	96§	144	79	−20.5
2-Dimethylaminofluorene	56	99	96	76	−1.0
	39	88§	32	84	−1.2
2-Nitrofluorene	48	77	74	83	−3.0
	62	84	57	—	—
2-Benzoylaminofluorene	49	69	71	93	—
	51	62	75	86	+2.0
2,7-Diacetylaminofluorene	58	28	29	91	+1.0
	61	35	49	—	—
2,2′-Diacetylamino-9,9′-bifluorylidene	57	11	7	—	−0.7
2,2′-Diacetylamino-9,9′-bifluoryl	50	0	—	—	—
	52	0	—	—	+4.5
		Group 2			
3-Acetylaminofluorene	98	78	72	98	−1.8
4-Acetylaminofluorene‖	73	90	80	—	−1.7
3-Iodo-2-acetylaminofluorene‖	74	32	51	—	+1.0
	99	29	28	0	−3.5
7-Iodo-2-acetylaminofluorene‖	72	47	58	—	−0.6
	100	94¶	92¶	93¶	−9.6¶
4-Acetylaminobiphenyl‖	76	91	68	—	−1.5
4,4′-Diacetylbenzidine	77	0	—	—	+3.0
Propylene glycol	—	—	—	—	+3.0
	—	—	—	—	+3.0

*The compounds were administered in propylene glycol in doses of 10 mg. 2-AAF or the molecular equivalent/100 gm. body wt. except where otherwise indicated.

†Conjugated material was estimated by subtracting the diazotizable material found without heating from the total diazotizable material recovered after heating for 1 hour in the presence of HCl of the reaction mixture.

‡The values for the diazotizable R-salt-coupling material recovered after compounds in group 1 were corrected for the lesser color density of the nitrite-reacting diazotizable metabolite present, assuming this to be 7-OH-2-AAF.

§Dosage was 20 mg. 2-AAF/100 gm. body wt. or the molecular equivalent.

‖The diazotizable R-salt-coupling material recovered from rats after these compounds of group 2 could not be corrected for possible differences in color density of metabolites giving positive nitrite tests.

¶Rat, no. 100, developed signs of middle-ear infection and failed to eat.

reaction, negative nitrite tests, and the histologic evidence supported the conclusion that the nodules in the rats consisted of unabsorbed diacetylaminobifluoryl and diacetylbenzidine respectively.

Table 3 shows that most of the diazotizable material recovered from the excreta of the rats after eight of the compounds in group 1 was from the urine. Most of the recovered material after 3- and 4-AAF, after 4-acetylaminobiphenyl, and from one rat after 7-iodo-2-AAF was also from the urine. The times when diazotizable material could no longer be detected in the urine varied, usually from 31 to 96 hours after administration, although small amounts were found for 5 days after 3-AAF, 4-AAF, and 7-iodo-2-AAF. Relatively small amounts of the recovered materials were present in the urine as free amines, table 3. However, the total diazotizable and nitrite-reacting material in each sample of urine from rat no. 99 (table 3), after 3-iodo-2-AAF, appeared to be present as free amines, giving the same amount of color before and after hydrolysis.

The last column of table 3 presents the average daily changes in body weight during the experimental period following administration of the compounds. The loss or gain in weight seemed to parallel the appetite of the individual rat. Food was always available but in most cases little was ingested during the first 24 to 48 hours following administration of the compound, after which varying amounts were consumed by individual rats. Two control rats lost weight during 24 hours following administration of propylene glycol but each gained an average of 3 grams daily during the period of 6 days in the metabolism cage.

Discussion

The carcinogenicity of compounds is difficult to compare quantitatively since many factors, biologic and chemical, influence the activity. Some of these influences can be controlled and varied, while others such as solubility and absorbability cannot be changed without modifying the compound and thus risk changing its metabolism. Such a comparison is often unsafe with studies made at different times in the same laboratory. This is true here, since for various reasons Morris has used different strains of rats and different diets and sometimes only one sex. It has become increasingly apparent that these factors play an important role in the kinds, the incidence, and the time of the appearance of induced tumors. If these sources of variation are neglected, however, the compounds of group 1 belong, according to carcinogenic potency, in roughly the same subgroups as those in which they were placed on the basis of the quantities of diazotizable and metabolized material estimated for the urine as 2-AF, 2-AAF, and 7-OH-2-AAF.

2-Di-AAF, 2-AAF, 2,7-di-AAF, and possibly 2-AF produce an early and/or usually a high incidence of tumors in the rat. 2-NF, 2-monomethyl-, 2-dimethyl-AF, and 2-BAF exert somewhat less or a delayed carcinogenic activity.

The results of this study indicate that the compounds in group 1, with the exception of 7-OH-2-AAF and 2,7-di-AAF, are metabolized in the rat in varying degrees to 2-AF, the amino group of which is conjugated (probably acetylated) before excretion. Thus, these compounds may owe their carcinogenicity to the ability to be converted to 2-AF, and the

mechanism of the activity would be the same as that of 2-AF. There is yet no experimental data to show whether the free amino or the conjugated amine or a metabolite of one of these forms is involved directly in the carcinogenic process. It is apparent, however, that the amount of carcinogen present in the animal at a given time would be influenced by the rate of the conversion of the various derivatives to 2-AF and that equimolar doses might not be so comparable as they are designed to be on that basis alone.

2-Aminofluorene, 2-acetylaminofluorene, and 2-diacetylaminofluorene.—The distribution of diazotizable material in the rat following a single intraperitoneal injection of 2-AF was investigated by Gutmann *et al.* (*11*) but no comparable data were included for comparison. In the present study, after equimolecular dosage, slightly greater amounts of diazotizable and of isotopic nitrogen were recovered from the urine following administration of 2-AAF compared with 2-AF. Carcinogenicity tests with rats, moreover, have indicated that 2-AAF is perhaps a little more active than the deacetylated derivative (*12, 13*). 2-Di-AAF followed closely the pattern of excretion of 2-AAF, and in the experience of Morris (*13, 14*) it is fully as potent a carcinogen for liver as 2-AAF.

7-Hydroxy-2-acetylaminofluorene.—7-OH-2-AAF has been reported to have a lower carcinogenic potency than the parent compound (*6, 15*). No evidence was obtained of its metabolism by the rat.

2-Methyl- and 2-dimethylaminofluorene.—The recovery of diazotizable material from the urine of the rats that received 2-methyl-AF (compound 5, table 1) is a little less than that with 2-AF (compound 4). The difference, however, would not be contradictory to the conclusions of Weisburger *et al.* (*16*), using methyl-C^{14}-AF, that the methyl group of this derivative is broken off from the molecule during metabolism. In the rat that received a dosage equivalent to 10 mg. 2-AAF per 100 grams of body weight, the dimethyl derivative led to the excretion of the same percentage of the administered compound as diazotizable material as did 2-monomethyl-AF. No increase in the percent of administered material recovered was obtained by doubling the dosage of 2-AAF or 2-methyl-AF and the recovery was decreased with increased dosage of 2-AF and 2-dimethyl-AF. Bielschowsky and Bielschowsky (*17*) found 2-dimethyl-AF less carcinogenic for the livers of rats than 2-methyl-AF and the monomethyl derivative less carcinogenic than 2-AAF in equimolecular amounts. Female rats of the Wistar strain, however, had a similar incidence of mammary cancers, whether 2-AAF, 2-methyl-AF, or 2-dimethyl-AF was fed. Miller, Miller, Sandin, and Rusch (*18*) reported the induction of liver tumors in Sprague-Dawley rats by 2-dimethyl-AF but they made no comparison with the potency of 2-AAF. Morris has found an equal incidence of hepatomas in female Buffalo rats after the feeding of 2-monomethyl and 2-dimethyl-AF (*8*).

2-Nitrofluorene.—Morris, Dubnik, and Johnson (*13*) obtained no liver tumors in 19 rats that received 2-NF by ingestion or by painting. A lower incidence of tumors was obtained (0.53 tumors per rat, none in the

liver) than with 2-AAF (1.2 per rat, of which 75 percent were in the liver). Miller *et al.* (*18*) reported the induction of liver tumors in rats following ingestion of 2-NF but no comparison was made of the carcinogenic potency to the liver with that of 2-AAF or 2-AF. The data presented here indicate that 2-NF is metabolized, in part at least, to 2-AF. The greater recovery from the urine of isotopic than diazotizable nitrogen (table 2) may be due to the presence of nondiazotizable, unmetabolized NF.

2-Benzoylaminofluorene.—The recovered material identified by spectrophotometric methods from the 2 rats that received 2-BAF may represent the same fractions that were responsible for the recovery by Gutmann and Peters (*19*) of 8 and 16 percent of the radioactivity of 2-BAF-9-C^{14} in the urine of rats during 96 hours and 11 days, respectively, following intramuscular administration. In the rat that was kept for 11 days 83 percent of the activity was found in the feces. Little, if any, unchanged 2-BAF was excreted in the urine, but indirect evidence was obtained of a glycuronide in the urine. After gastric administration, urinary recoveries of 25 and 15 percent, respectively, of the potential 2-AAF administered as 2-BAF were obtained and, of that recovered, 56 and 66 percent was present in a form that behaved spectrophotometrically like 7-OH-2-AAF, which is present in urine largely as the glycuronide (*4*, *5*), observations which are in agreement with Gutmann *et al.* The recoverable diazotizable material from both urine and feces in this study was excreted within 72 hours after administration; the first 24-hour-feces sample contained more than did the 24- to 48-hour sample and the 48- to 72-hour sample contained a barely detectable amount. If the excretory behavior of orally administered 2-BAF resembles that of intramuscularly administered 2-BAF, it is possible that small amounts of diazotizable material were excreted in the feces over a period of days but could not be detected by the colorimetric method of analysis. Morris (*8*) has found that 2-BAF is a weaker carcinogen than 2-AF or 2-AAF under similar experimental conditions. The weaker carcinogenicity may possibly be due to failure of absorption and to final excretion of large amounts in the feces.

2,7-Diacetylaminofluorene.—Pinck (*20*) predicted that 2,7-di-AAF would be more carcinogenic than 2-AAF because the presence of a substituent in the 7-position would block oxidation to 7-OH-2-AAF. 2,7-Di-AAF is a potent carcinogen especially in the production of intestinal tumors (*21*). The recovery of diazotizable material from the urine was less from 2,7-di-AAF than from 2-AAF, with half of the administered compound accounted for in the feces. There was no more nitrite-reacting material detected in the feces than would be expected by interference in the color test by the amount of unmetabolized compound estimated to be present. If there is any connection between the large amount of material excreted by the intestine and the high incidence of intestinal tumors, the presence of a substituent group in the 7-position may be implicated in the carcinogenesis. The presence of 32 and 29 percent of nitrite-reacting substance in the urine of the total diazotizable-R-salt-coupling material recovered suggests the possible presence of a metabolite. Unless such a

metabolite has a lesser color density than 7-OH-2-AAF in the nitrite test, the oxidation of 2,7-di-AAF, if it occurs at all, must be less than that of 2-AAF.

2,2'-Diacetylamino-9,9'-bifluorylidene.—The bifluorylidene derivative was poorly absorbed and was found to induce few tumors (*21*).

Fluorene.—There is agreement in the literature that fluorene does not induce tumors under the experimental conditions that have been used for testing the 2-amino derivatives (*22*, *8*). Neish (*23*) isolated 2-hydroxyfluorene and the glycuronide of this phenol from the urine of rabbits fed fluorene, but these metabolites lacking the amino substituent group are negative in the nitrite test. Thus the failure to find any trace of fluorene metabolites by the methods used here was not surprising.

3- and 4-Acetylaminofluorene.—3- and 4-AAF of group 2 are weakly carcinogenic to Buffalo strain female rats (*8*, *10*, *24*). These derivatives, however, led to the excretion of as much diazotizable material in the urine as did 2-AF and other related compounds that are decidedly more carcinogenic. Considerable amounts of nitrite-reacting material, which could not be 7-OH-2-AAF, were also excreted. The 2-OH-3-AAF that was isolated after 3-AAF administration was from an ether extract of the urine (*10*). However, 86 percent of the detectable material of the urine of the rat in this study remained in the urine after repeated ether extraction, indicating that most of the 2-OH-3-AAF in the urine is preferentially water soluble. The data for the nitrite-reacting material recovered from a rat following administration of 4-AAF indicated that 1-OH-4-AAF, if present, is not the only nitrite-reacting metabolite. Another, or other metabolites that yield greater amounts of color in this reaction would be expected to be present.

4-Acetylaminobiphenyl.—Miller *et al.* (*18*, *25*) report this compound to be a less active carcinogen for the liver and some other sites, but to be as active a mammary carcinogen as the related 2-AAF. According to the percentage of administered material detectable in the urine in the diazo test and the evidence of its metabolism, this compound would belong in the second subgroup of compounds mentioned above.

Iodo derivatives of 2-acetylaminofluorene.—7-Iodo-2-AAF induces few, if any, tumors while 3-iodo-2-AAF is a potent carcinogen, particularly in inducing ear tumors (*8*). The suggestion that an active hydrogen on the 7-carbon of 2-AAF is necessary to carcinogenicity needs further investigation as to the stability of the substituent groups in the 7-position, since 7-fluoro-2-AAF has been found by Miller *et al.* (*18*) to be a more active hepatic carcinogen than the parent compound. More color appeared in the urine of the rats in the nitrite test after administration of either iodo derivative than would be expected if the diazotizable material were present as unmetabolized compounds. Furthermore, 100 percent of the urinary material recovered from the urine after 3-iodo-2-AAF was present as free amine, whereas 93 percent of that recovered after 7-iodo-2-AAF was conjugated with respect to the amino group so that heat hydrolysis was necessary for diazotization. This observation seems worthy of

further study since the difference in metabolic behavior is probably related to the position of the substituent groups and may be pertinent to the difference in the carcinogenic potencies.

The bifluoryl and benzidine compounds.—The failure to find tumors in rats after oral administration of 2,2′-diacetylamino-9,9′-bifluoryl (*21*) and of only one tumor with 4,4′-diacetylbenzidine (*8*) may be explained by the data presented in this study showing the nonabsorbability of these water insoluble compounds when administered by stomach tube or by intraperitoneal injection, although the carcinogenicity studies of the benzidine compound may have been complicated by toxic effects.

Mechanism of carcinogenesis.—Not many theories for the mechanism of carcinogenesis by 2-AAF have been postulated. Protein binding as a necessary step in the carcinogenic process has been proposed by Miller and Miller (*26*), with experimental evidence in support of the theory for diazo-dye carcinogens and for several hydrocarbons. Binding of N^{15} by proteins of the liver following administration of 2-AAF-N^{15} (*5*), and of radioactivity after 2-AAF-9-C^{14} (*27*) have been demonstrated in this laboratory. There is as yet no evidence of the nature of the substance bound nor for the site of binding with AAF. Pinck (*20*) offered a working hypothesis of carcinogenesis involving for 2-AAF the reactivity of the hydrogen of the methylene group and the potential production of a dehydrogenated dibiphenylene-ethylene which in turn could condense with the cell substance.

A third proposal, by Clayson (*28*), that aromatic amines are carcinogenic because of their conversion to *ortho*-hydroxy amines, is based on the experimental studies of Bonser, Clayson, and associates (*29*) supporting the conclusion that 2-amino-1-naphthol is the active carcinogen in the production of bladder tumors by β-naphthylamine. As yet there is no evidence that 1-hydroxy or 3-hydroxy-2-AAF are or are not active carcinogens. Based on the colorimetric methods as indicating that 7-OH-2-AAF is quantitatively a major metabolite of 2-AAF in the rat and that 2-OH-3-AAF is a major metabolite of 3-AAF, it would seem that there is no general relation between the amount of *ortho*-hydroxy amine formed and carcinogenicity of the parent acetylaminofluorenes, since relatively small amounts of 1- and 3-OH-2-AAF have been found to be excreted following administration of the strongly carcinogenic 2-AAF, whereas a large portion of the relatively noncarcinogenic 3-AAF is excreted as 2-OH-3-AAF.

There appears to be some correlation between the degree of carcinogenicity (especially for the liver) to rats of the substances investigated in group 1, table 1: 1) the amount of diazotizable-R-salt-coupling material excreted in the urine; and 2) the formation of nitrite detectable metabolites which behave like 7-OH-2-AAF.

At this time it is not known whether 2-AAF is the carcinogen or whether it must be metabolized to an active form. The experimental evidence cited above rules out the chance that 7-OH-2-AAF is the active carcinogen. Chemical-biological reactions involved during the oxidation of 2-AF

or 2-AAF to the hydroxylated product(s) rather than the metabolite itself might be related to the ability to induce neoplasia. The amount of diazotizable and nitrite-reacting materials excreted may be only an indication of the degree to which the individual compounds are metabolized by the rat, and bear no relation to the ability of these chemicals to transform normal cells to cancer cells. The analytical results at this time do not permit a general assumption that the amount of metabolism measured by the nitrite test is related to the degree of carcinogenicity. Thus, 3- and 4-AAF are weakly, if at all, carcinogenic and yet in their behavior in the nitrite test they give evidence of being metabolized to a large extent, while the potent carcinogen, 2,7-di-AAF, shows little evidence of metabolism by the same test.

Another contradictory observation is that 2-AAF administered to the guinea pig, in the same manner, and dosage, on a weight basis, as to the rat, shows the same excretory behavior as in the rat, except that more (75 percent) of the urinary diazotizable-R-salt-coupling material reacts as 7-OH-2-AAF (*5*). Numerous unsuccessful attempts have been made to induce tumors in the guinea pig by 2-AAF. The chemical has been found in this laboratory to be toxic to guinea pigs so that survival may not have been long enough for tumors to appear (*8*).

Finally, since oxidation of aromatic amines and other compounds to phenolic derivatives appears to be a common means of metabolism, the most acceptable view is that these studies may simply indicate the comparative absorbability of the individual compounds and the percentage of the administered dose that is metabolized by conversion to catabolites that are detectable by the methods of analysis used.

Summary

Ten derivatives of 2-aminofluorene (2-AF) and seven substances with closely related structures, dissolved in propylene glycol, were administered to rats by stomach tube in equimolecular amounts. The urine and feces were collected and analyzed 1) for diazotizable-R-salt-coupling material and 2) for metabolites that could be detected by the so-called nitrite test.

The urinary excretion of diazotizable-R-salt-coupling material was equal to 50 to 80 percent (average 63 percent) of the diazotizable or potentially diazotizable materials from 2-AAF, 2-AF, and 2-di-AAF and a very small portion was in the feces. A large fraction of the diazotizable material recovered after administration of each of these three compounds reacted in the nitrite test for 7-OH-2-AAF, thus proving that they had been metabolized at least to this extent.

Somewhat smaller fractions of the administered diazotizable or potentially diazotizable compounds were recovered from the urine by the R-salt-coupling method following administration of 2-monomethyl-AF, 2-dimethyl-AF, 2-NF, 2-BAF, 2,7-di-AAF, 3-AAF, 4-AAF, and 4-acetylaminobiphenyl. With this last mentioned group of fluorene derivatives and the biphenyl compound, there was also evidence of a considerable production of nitrite-reacting metabolites but very little of the recovered material was present in the feces, except after 2,7-di-AAF. The nitrite-

reacting metabolites of the 2-amino derivatives were assumed to be 7-OH-2-AAF. That from 3-AAF was estimated as 2-OH-3-AAF. The identities of metabolites from 4-AAF and 4-acetylaminobiphenyl are unknown, but the reacting excess material when estimated as 1-OH-4-AAF suggests that this derivative cannot be the sole metabolite of 4-AAF responsible for the nitrite test.

3- and 7-Iodo-2-AAF resulted in the excretion of diazotizable-R-salt-coupling material in the urine equal in amount to that of some of the preceding group. The nitrite test was unsatisfactory for the iodo derivatives because the substituent iodine is unstable in the acid medium of the analytical procedures. However, the excreted material in the urine reacting in this test following administration of 3- and 7-iodo-2-AAF shows a significant increase in the amount of color produced over that expected from the total diazotizable material present as the unmetabolized compound. This increase could indicate metabolism of the iodo derivatives to metabolites that react directly in the nitrite test, or to metabolites in which the substituent iodine is even more unstable in the acid medium of the tests than that of the administered compounds.

2,2′-Diacetylamino-9,9′-bifluorylidene was excreted in great part by the large intestine but there was evidence of some metabolism of the small amount of diazotizable material excreted by the kidneys.

2,2′-Diacetyl-9,9′-bifluoryl and 4,4′-diacetylbenzidine were detected only in the feces after gastric administration and only in the peritoneal cavity after intraperitoneal administration, and there was no evidence of metabolism of the recovered diazotizable materials.

None of the compounds (except the bifluoryl derivative from the feces of one rat) were accounted for quantitatively by the two spectrophotometric methods of analysis, but with isotopic nitrogen approximately 100 percent of the N^{15} administered as 2-AF, 2-AAF, and 2-NF was recovered.

The difficulties in establishing any relation between the excretory behavior of the compounds studied and their carcinogenicity have been considered.

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Enzyme Changes Induced in Normal and Malignant Tissues With Chemical Agents. IV. Effect of *Alpha*-Peltatin on Glucose Utilization by Sarcoma 37 and on the Adenosinetriphosphatase, Hexokinase, Aldolase, and Pyridine Nucleotide Levels of Sarcoma 37[1,2]

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Previous communications from this laboratory (*2–4*) on the mechanism of action of tumor-damaging compounds showed that a marked decrease in cytochrome oxidase activity, cytochrome c, succinoxidase activity, and respiration of tumor tissue homogenates or slices occurred soon after the *in vivo* administration of these drugs. The loss of enzyme activity occurred in about 2 hours and the maximum rate of drop in activity was attained in about 4 to 8 hours after injection.

To supplement these findings on the effect of these drugs on enzyme mechanisms, work on tumor tissue was extended to a study of the ability of tissues from treated animals to utilize glucose and an investigation of the drug action on enzyme systems involved in the phosphorylation of glucose.

The data presented here deal with glucose utilization by tumor tissue from animals previously injected with *alpha*-peltatin and furnish information on enzymes concerned with diminished glucose utilization.

Materials and Methods

$CAF_1[(BALB/c \times A)F_1]$ mice bearing 6-day-old intramuscular implants of Sarcoma 37 were employed. The compound used was *alpha*-peltatin (NCI-1074) isolated from podophyllin (*5*). The procedure for preparing and injecting solutions of the compound into mice has been described (*2*). The animals were killed 4 to 6 hours after a single injection. Tumor tissue, freed from grossly visible necrotic areas, was homogenized with 2 volumes of ice-cold distilled water. The material was strained through cheesecloth and the filtrate used at once for the measurement of glucose consumption. Throughout these operations the tissue was kept at 0° C.

For each test reaction, 0.5 ml. of tumor homogenate prepared from two to four tumor-bearing animals was added to the Warburg vessels containing glucose (0.6 ml. of a 0.2% solution), $MgCl_2$ (0.1 ml. of a 0.005 *M*

[1] Received for publication December 14, 1954.
[2] Presented in part at the fall meeting, American Association for Pharmacology and Experimental Therapeutics, September 6 to 8, 1954, Charlottesville, Va. (*1*).
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solution), $NaHCO_3$ (0.5 ml. of a 0.2% solution), phosphate buffer (0.9 ml. of a 0.02 *M* solution, *p*H 7.8), coenzyme I, Sigma 90-percent purity (0.2 ml. of a 0.00016 *M* solution), and ATP [5] in excess (1.4 mg. 7 minute P per sample) and incubated at 37° C. for 30 minutes in an atmosphere of 95-percent N_2 and 5-percent CO_2. After 0 and 30 minutes of incubation, 1 ml. of the mixture was run into 3 ml. of distilled water followed by 0.75 ml. each of 0.3 *N* $Ba(OH)_2$ and 5-percent $ZnSO_4 \cdot 6H_2O$. The estimation of glucose in the filtrate was carried out according to the method of Nelson (*6*). In some experiments, higher concentrations of DPN up to 0.006 *M* were used.

Measurement of CO_2 production at 10-minute intervals was carried out in Warburg vessels using 0.5 ml. of tumor homogenate and 2.6 ml. of Krebs-Ringer bicarbonate solution with and without glucose incubated at 37.5° C. in an atmosphere of 95-percent N_2 and 5-percent CO_2.

Tumor-bearing mice were injected with *alpha*-peltatin (20 μg./gm.) and sacrificed after different time intervals to determine the effects on ATPase, aldolase, hexokinase activity, and on DPN content of the tumor.

ATPase activity of the control and treated tumor tissue was determined by the method of DuBois and Potter (*7*), which estimates inorganic phosphate released from ATP by ATPase present in the tissue.

Aldolase activity of the tissues was determined by the method of Sibley and Lehninger (*8*) with 0.05 *M* fructose-1, 6-diphosphate at *p*H 8.6 as substrate.

The method employed for determining hexokinase activity of tumor was similar to the one reported by Slein, Cori, and Cori (*9*).

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}.$$

$$\text{Glucose-6-phosphate} + \text{TPN} \xrightarrow{\text{zwischenferment}} \text{6-phosphogluconate} + \text{reduced TPN}.$$

The reaction mixture contained glucose (0.06 ml. of a 0.2% solution), $MgCl_2$ (0.1 ml. of a 0.005 *M* solution), $NaHCO_3$ (0.3 ml. of a 0.2% solution), phosphate buffer (1.0 ml. of a 0.02 *M* solution, *p*H 7.8), ATP (0.04 ml. of a 0.112 *M* solution), TPN, Sigma 80 percent (0.5 ml. of a 0.00175 *M* solution), zwischenferment, Sigma (0.6 mg. in 0.1 ml. of 0.1% $NaHCO_3$), KCN (0.1 ml. of a 0.0004 *M* solution), tumor homogenate and water to make 3 ml. The blank contained the same components except for glucose. Measurement of optical density at 340 millimicrons was made in a Beckman DU spectrophotometer at room temperature, with readings taken at 1-minute intervals. The rate of reduction of TPN was linear with time for about 8 minutes and was directly proportional to the concentration of the enzyme. The results were expressed in terms of number of micromoles of TPN reduced per minute with the use of a molecular extinction coefficient of 6.22×10^6 sq. cm. per mole of reduced TPN.

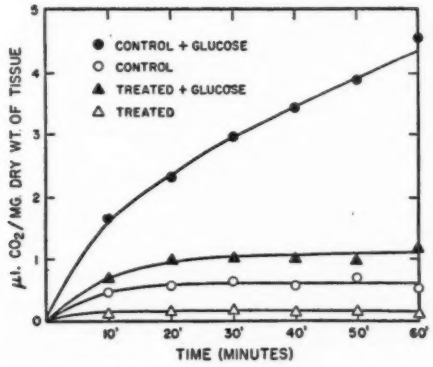
[5] For the sake of brevity and convenience, the following abbreviations have been employed: ATP=adenosinetriphosphate; ATPase=adenosinetriphosphatase; pyridine nucleotide=diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN); ADP=adenosinediphosphate.

Pyridine nucleotide was estimated by the fluorometric acetone condensation method employed by Robinson, Levitas, Rosen, and Perlzweig (*10*). Tissues were removed and immediately immersed in a beaker containing 2-percent nicotinamide and 15 to 20 mg. $Ce(SO_4)_2$ to prevent any loss of the coenzyme. They were homogenized and filtered; appropriate dilutions were then made to suit the sensitivity of the fluorometer. DPN used in the procedure was of 90-percent purity (Sigma). The results are expressed as microgram of DPN per gram of fresh tissue.

All these determinations were carried out in duplicate.

Results

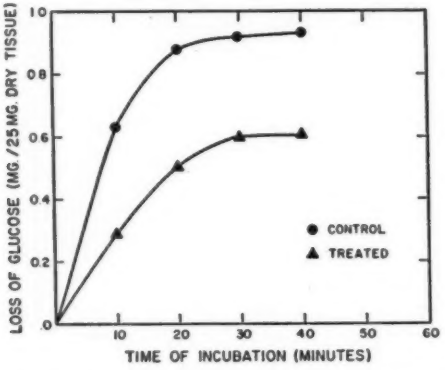
Anaerobic glycolysis of Sarcoma 37 from mice injected with alpha-*peltatin.*—Text-figure 1 shows rate of glycolysis, with and without added glucose, of Sarcoma 37 from mice 5 hours after the injection of 20 μg. per gram of *alpha*-peltatin and also from uninjected controls. In the absence of glucose, carbon dioxide output was only slightly greater in the control tumor. However, when glucose was added to the medium, the CO_2 output was markedly greater in the control tumor. This finding led to further work on glucose uptake by these tissues.



TEXT-FIGURE 1.—Rate of anaerobic glycolysis of Sarcoma 37 from mice injected with *alpha*-peltatin.

Glucose disappearance in Sarcoma 37 from mice injected with alpha-*peltatin.*—Tumor homogenates from drug-treated and from untreated mice were examined to find the rate of glucose disappearance with time of incubation. Text-figure 2 shows that the maximum amount of glucose utilized by the tissue homogenates was reached within 30 minutes of incubation; the rate of glucose utilization was slower in treated tissue. Results on glucose disappearance in tumor homogenates from several drug-treated and from untreated mice are summarized in table 1. The tumor homogenates from drug-treated mice showed about 40-percent lower ability to utilize glucose than those from the untreated mice. Whether the difference in glucose utilization by the tumor homogenates

from the drug-treated and untreated animals was a reflection of the effect of the drug on the hexokinase activity of the tumor was next studied by determining the hexokinase activity of the tumor homogenates from injected animals.



TEXT-FIGURE 2.—Rate of glucose utilization by Sarcoma 37 from mice injected with *alpha*-peltatin.

TABLE 1.—*Glucose disappearance in tumor homogenates from mice injected with* alpha-*peltatin (20 μg./gm.)*

Experiment number	Control tumor*	Treated tumor*
1	0. 96	0. 62
2	. 97	. 62
3	. 93	. 64
4	. 95	. 62
5	1. 00	. 58
6	. 98	. 64
Average	. 97	. 62

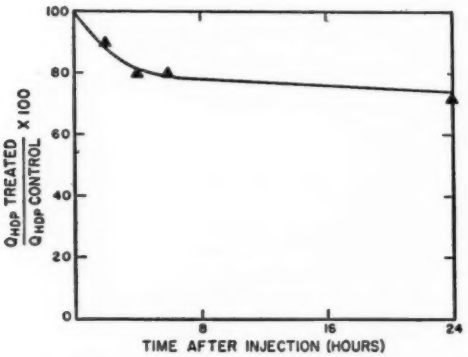
*Results expressed in mg. of glucose utilized by 25 mg. of dry tissue after 30 minutes of incubation.

TABLE 2.—*Effect of injection of* alpha-*peltatin on hexokinase activity of Sarcoma 37*

Time after injection* (*hours*)	Residual activity of tumor† (*percent*)
2	92
4	109
6	98
24	21

*20 μg./gm. of *alpha*-peltatin injected subcutaneously.
†μM TPN reduced per minute by 1 mg. tissue (dry) $\frac{\text{treated tumor}}{\text{control tumor}} \times 100$. Range of values for control tumor: 0.0057 to 0.0071.

Effect of alpha-*peltatin on hexokinase, aldolase, and adenosinetriphosphatase activity of Sarcoma 37.*—Results on the hexokinase activity of Sarcoma 37 are given in table 2. The hexokinase activity of the tumor remained essentially unaltered for 6 hours after the injection of the drug; after 24 hours about 80 percent of the activity was lost. Text-figure 3 gives the results obtained with single subcutaneous injection of 20 μg. per gram of *alpha*-peltatin on aldolase activity of Sarcoma 37. Nearly 80 percent of the enzyme activity was retained until 8 hours after injection; further loss in activity during the next 16 hours was very small. The effect of the compound on the enzyme activity was barely noticeable when compared with that on other systems of enzymes of Sarcoma 37, such as cytochrome oxidase activity (*2*).



TEXT-FIGURE 3.—*In vivo* effect of 20 μg. per gram of *alpha*-peltatin on aldolase activity of Sarcoma 37. Units of aldolase expressed as Q_{bdp}=cu. mm. of hexose diphosphate split per mg. dry weight of tissue per hour at 37.5° C. Control value range for Sarcoma 37=49.0 to 51.5.

Table 3 illustrates the residual adenosinetriphosphatase activity of tumors and of livers from mice treated with the drug. Here again, more than 80 percent of the enzyme activity of both the tissues was left unimpaired for 24 hours after the injection of the compound. It seems therefore that the diminished capacity of the treated tumor to utilize glucose following injection of the drug is not to be attributed to the relatively small loss in aldolase or in adenosinetriphosphatase activity.

Effect of alpha-*peltatin on pyridine nucleotide level of Sarcoma 37.*—Diphosphopyridine nucleotide level in the tumors from mice treated with the drug was next investigated. These results are given in table 4. There was a marked loss (about 50%) in the coenzyme activity of the tumors within 2 hours of administration of the compound, and about 65 percent of the activity was lost within 6 hours of treatment. The diminished DPN level was considered in relation to the lowered glucose uptake by the treated tumors. Experiments on glucose uptake were then repeated with excess DPN in the medium.

TABLE 3.—*Effect of injection of* alpha-*peltatin on adenosinetriphosphatase activity of Sarcoma 37 and of liver*

Time after injection* (*hours*)	Residual activity†	
	Tumor (*percent*)	Liver (*percent*)
2	96	88
4	81	92
6	90	99
24	89	99

*20 μg./gm. of *alpha*-peltatin injected subcutaneously.
†$\frac{\text{Units with treated tumor}}{\text{Units with control tumor}} \times 100$ unit values (μg. P per mg. fresh tissue) from untreated mice. Sarcoma 37: 10.1–10.5. Liver: 13.6–15.2.

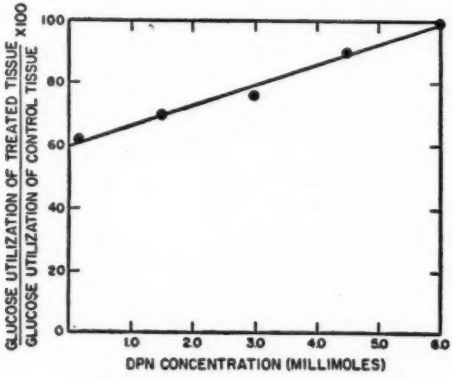
TABLE 4.—*Effect of subcutaneous injection of* alpha-*peltatin on the pyridine nucleotide content of Sarcoma 37*

Time after injection* (*hours*)	Pyridine nucleotide content† of Sarcoma 37						
	1	*2*	*3*	*4*	*5*	Average	Percent of control‡
Control	229	248	220	266	272	247	100
2	121	140	113	117	145	127	51
4	98	107	78	105	108	99	40
6	79	110	76	85	.....	88	35
Control	205	180	223	224	180	202	100
24	63	72	51	38	89	63	31

*20 μg./gm. of *alpha*-peltatin injected subcutaneously.
†Expressed as μg. DPN/gm. wet tissue; the values in the columns 1 to 5 are determinations on separate animals.
‡$\frac{\text{Average treated}}{\text{Average control}} \times 100$.

Glucose disappearance in tumor homogenate in presence of excess DPN.—Although the low DPN concentration in the substrate mixture, in the experiments described in the beginning, was sufficient for glucose utilization by control tumor homogenate, maximum utilization of glucose was not obtained with tissues from the treated tumors. With a gradual increase in DPN concentration in the medium, treated tumor homogenates showed an increased ability to utilize glucose up to a DPN concentration of about 0.006 *M*, at which level the glucose utilized by control and treated tumor was alike (text-fig. 4). A similar increase of DPN in the substrate of control tumor tissue (0.00016 to 0.006 *M*) did not produce any significant change in glucose utilization.

The rate of glucose disappearance in treated and control tumor homogenate was measured at a high concentration of DPN in the medium. It was found (text-fig. 5) that the rate of glucose uptake by both tumor homogenates was similar after each period of incubation. This is again illustrated in table 5 when tumors from several injected and noninjected mice were incubated with glucose for 30 minutes.

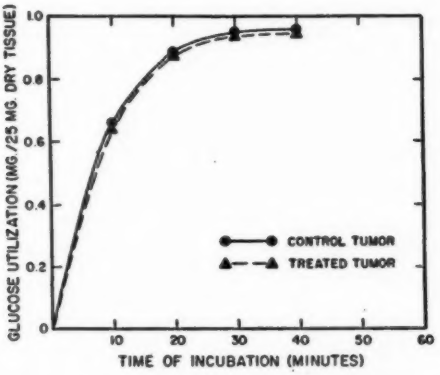


TEXT-FIGURE 4.—Effect of different concentrations of DPN on glucose utilization by Sarcoma 37 from mice injected with *alpha*-peltatin.

TABLE 5.—*Glucose disappearance with excess DPN in tumor homogenates from mice injected with* alpha-*peltatin*

Number	Control*	Treated*
1	0. 91	0. 90
2	. 94	. 90
3	. 91	. 91
4	. 93	. 91
5	. 93	. 93
6	. 92	. 92
Average	. 92	. 91

*Results expressed in mg. of glucose utilized by 25 mg. of dry tissue after 30 minutes of incubation.



TEXT-FIGURE 5.—Effect of excess DPN on rate of glucose utilization by Sarcoma 37 from *alpha*-peltatin injected mice.

Discussion

The data presented here show that tumors removed from animals 4 to 6 hours after the injection of *alpha*-peltatin had a lower rate of anaerobic glycolysis than that of tumors from untreated animals. When glucose was added to the substrate the difference in anaerobic glycolysis between the control and the treated tumors increased. The ability of the treated tumor to utilize glucose was about 40 percent less than that of the control tumor.

As has been stated by previous workers (*11–13*), glycolysis in normal or tumor tissue is inhibited by homogenization but can be restored by the addition of various cofactors. In our experiments, such cofactors were made available in the reaction for determining glucose utilization by the tissues.

The first step in glycolysis where loss in activity would be reflected in a low glucose utilization might occur in diminished hexokinase activity. Determinations of hexokinase activity of tumor tissue from the injected animals showed that enzyme activity remained practically constant over a period of 6 hours after the administration of the drug. It was only after 24 hours, after the tumors had become necrotic, that the bulk of activity was lost.

Similarly, the changes observed in other enzymes such as adenosinetriphosphatase or aldolase could not entirely account for the low glucose utilization by tumors 4 hours after injection of compound.

According to Weinhouse and others (*14*) DPN has been found to be necessary for the activation of the oxidation of endogenous substrates of homogenates of tumors. In the present experiments the level of DPN in the substrate medium was enough for the utilization of glucose by homogenates of control tumors but was insufficient for homogenates of treated tumors. With an increased concentration of DPN to 0.006 *M* in the medium, the ability of the homogenate of treated tumors to utilize glucose was similar to that of untreated tumors; increased levels of DPN above 0.00016 *M* did not increase glucose utilization in untreated tumors.

A marked drop in endogenous pyridine nucleotide level of the tumor tissues occurred shortly after administration of the drug. Since it has been postulated (*14*) that DPN in tumor tissue is very loosely bound to cell particulates, it is possible that such losses in the endogenous pyridine nucleotide levels of the tumors may require greatly increased levels of DPN in the medium to permit glycolysis to proceed at rates comparable to those of untreated tumors.

Summary

1) Homogenates of Sarcoma 37 obtained from mice a few hours after the administration of a single subcutaneous dose of 20 μg. per gram of *alpha*-peltatin showed about 40-percent decreased ability to utilize glucose anaerobically than homogenates from untreated mice.

2) With increasing DPN concentration in the medium, *alpha*-peltatin-treated tumor homogenates showed an increased ability to utilize glucose,

reaching a maximum at a DPN concentration of 0.006 *M*. Similar increases in DPN levels did not increase the ability of control tumor to utilize more glucose over that observed with the lower DPN concentration (0.00016 *M*) in the medium. At a DPN concentration of 0.006 *M* the glucose uptake by control and treated tumor was the same.

3) A single subcutaneous injection of 20 μg. per gram of *alpha*-peltatin in mice bearing Sarcoma 37 produced a marked drop in pyridine nucleotide level of tumor soon after the administration of the drug.

4) Only small changes in adenosinetriphosphatase, aldolase, and hexokinase activities were observed during the period (4 to 6 hours) in which the glucose utilization and pyridine nucleotide level exhibited marked decreases.

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Neoplasms in Mice Inoculated with Cell-Free Extracts or Filtrates of Leukemic Mouse Tissues. II. Leukemia in Hybrid Mice Produced by Cell-Free Filtrates[1]

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The incidence of spontaneous, acute lymphatic leukemia in strain C3H mice and in the C3Hf line of this strain has been reported to be extremely low (*1*). The leukemia incidence in inbred mice is known to be dependent on the genetic constitution of the strain, and the susceptibility to "leukemogenic" substances, either artificially applied or as naturally occurring agents, may also be determined by genetic factors. Kirschbaum and Mixer (*2*) studied the induction of leukemia in 8 inbred strains of mice varying in susceptibility to the spontaneous disease and found that mice of the C3H strain, which had a low incidence of spontaneous leukemia, were relatively resistant to the carcinogens tested for "leukemogenic" activity. In their experiments, the C3H mice were "refractory to the leukemogenic action of methylcholanthrene but probably somewhat susceptible to the leukemia-inciting action of X rays and estradiol dipropionate." Gardner *et al.* (*3*) also found strain C3H mice somewhat susceptible to the leukemia-inciting action of estrogens. Mice treated with high doses of estrogens showed an incidence of approximately 15-percent lymphomas, while in X-ray treated C3Hf mice free of the mammary tumor agent Lorenz *et al.* (*4*) found a 36-percent incidence of lymphomas of various types. These mice were exposed to chronic total-body irradiation with a mean accumulated dose of 3,570 r.

In a preceding communication (*5*), it was shown that newborn C3H mice were relatively resistant to any leukemia-inciting properties resulting from inoculation with tissue extracts or filtrates derived from leukemic strain AKR mice, in contrast to the results of Gross (*7–11*). This resistance to a possible naturally occurring agent, which may be present in cell-free extracts of leukemic mouse tissues, could be due to the genetic constitution of this strain of mice. Therefore, in order to demonstrate the action of a leukemia-producing agent, it would be necessary to increase the susceptibility of this low-leukemia strain by crossing it with a high-leukemia strain.

In 1952, Law (*1*) was able to increase the incidence of spontaneous leukemia in (C3Hf × AKR)F_1 hybrid mice from 27.7 percent at 14

[1] Received for publication December 10, 1954.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

months in the control animals to 60.9 percent in the experimental animals by implanting fragments of normal thymic tissue from 4- to 5-week-old strain AKR mice. He concluded that normal AKR thymic tissue in some manner induced leukemia in the (C3Hf × AKR)F_1 hosts.

In the work to be reported here, it has been possible to demonstrate a leukemia-producing agent in the tissues of leukemic AKR mice by inoculating newborn (C3Hf × AKR)F_1 hybrids with cell-free filtrates prepared from extracts of these tissues, and this observation has been extended by demonstrating a leukemia-producing agent in the tissues of the F_1 hybrids developing leukemia as a result of these inoculations.

Materials and Methods

The mice used were of the same origin as those described in the preceding paper (*5*), with the exception of some normal AKR mice obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and some normal AK-n mice obtained from Dr. Ludwik Gross, Veterans Administration Hospital, Bronx, New York. In the first series of experiments both the C3Hf females and the AKR males were obtained from Dr. Lloyd Law, National Cancer Institute, Bethesda, Maryland. In the second series of experiments the C3Hf females were obtained from Dr. George Jay of the National Institutes of Health, and these were mated with AKR males obtained from Dr. Law, Dr. Gross, and from Bar Harbor. Controls were kept for each group of hybrids to determine the normal incidence of leukemia and the date of its appearance in each group; only first litters were used in all instances. The animals were kept under the conditions previously described (*5*).

For the first series of experiments filtrates were prepared from tissues of strain AKR mice bearing spontaneous leukemias; for the second series of experiments filtrates were prepared from transplanted AKR leukemias and from the tissues of (C3Hf × AKR)F_1 hybrids that had developed leukemia after inoculation with the AKR filtrates in the first experiments. In each instance the enlarged thymus, mesenteric and peripheral lymph nodes, liver, spleen, and transplanted tumor (if present) were removed and homogenized in a Potter-Elvehjem homogenizer, and enough physiologic saline was added to make a 20-percent suspension. This suspension was centrifuged for 15 minutes at 3,000 r.p.m. (1,400 × g) at 0° C., and the supernate was removed and centrifuged for 10 minutes at 9,500 r.p.m. (7,000 × g) at 0° C. The supernate was then filtered either through a Morton glass filter of fine porosity, which had been shown to hold back cultures of *Escherichia coli*, or a Selas filter of #03 porosity. Newborn hybrid mice, 3 to 12 hours old, were inoculated subcutaneously with 0.1 ml. of the resulting cell-free filtrate, and the same filtrates were used to inoculate newborn C3H and C3Hf mice in the same age range. For controls, hybrid mice from the same breeders were left either uninoculated or inoculated with 0.1 ml. of filtrate that had been heated at 80 to 90° C. for one hour. One mouse with leukemia was sacrificed for each

experiment with the exception of experiment 6, table 2. In this experiment part of the homogenate used in experiment 5 was frozen and thawed and allowed to stand for 72 hours at 4° C., as described earlier (*5*), before centrifugation and filtering.

Results

Leukemia-Producing Agent in Leukemic AKR Tissues

The results of the first series of experiments are summarized in tables 1–3.

It will be noted (table 3) that only one strain C3H mouse developed leukemia. This mouse developed a generalized leukemia when the animal was 14 months old, and on transplant it grew only in C3H mice. The F_1 hybrids receiving the same filtrates (tables 1 and 2) began to develop

TABLE 1.—*Leukemia in (C3Hf × AKR)F_1 hybrid mice inoculated with unheated and heated filtrates from tissues of leukemic AKR mice (expt. 1–7)*

Filtrate	Number of mice inoculated	Number surviving 3 weeks or more	Number developing leukemia	Number developing leukemia each month of age										
				3	4	5	6	7	8	9	10	11	12	13	14
Unheated	61	45	27	1	2	2	5	4	7	1	1	1	3	0	0
Heated (control group)	54	44	10	0	0	0	0	0	0	2	4	2	1	1	0

TABLE 2.—*Leukemia in (C3Hf × AKR)F_1 hybrid mice inoculated when newborn with filtrate from leukemic AKR tissues*

Expt. No.	Number of mice inoculated	Sex	Number surviving 3 weeks or more	Number developing leukemia	Ages at which leukemia appeared (*months*)
1	4	♀	3	3	6, 6.5, 12
	2	♂	2	1	8
2	3	♀	3	3	8.5, 9, 10
	3	♂	3	2	8, 12
3	3	♀	3	2	4, 12
	4	♂	4	4	6, 7, 7, 8
4	4	♀	1	0	—
	3	♂	3	2	5, 7
5	6	♀	4	0	—
	9	♂	5	2	3, 8
6	9	♀	5	3	5, 6, 8
	5	♂	3	0	—
7	4	♀	4	4	4, 6, 7, 11
	2	♂	2	1	8
Total	33	♀	23	15	
	28	♂	22	12	

TABLE 3.—*Leukemia in C3H and C3Hf mice inoculated when newborn with the leukemic AKR filtrates used in table 2*

Expt. No.*	Strain of mouse inoculated	Number inoculated	Number surviving 3 weeks or more	Number developing leukemia
1	C3Hf	9	7	0
2	C3H	6	4	1†
3	C3Hf C3H	6 10	0 0	0 0
5	C3Hf	12	4	0
6	C3Hf	7	6	0
7	C3H	7	6	0
Total		57	27	1

*Experiment numbers correspond to those in table 2. The same filtrates were used for each pair or corresponding experiments.

†Leukemia at 14 months of age.

leukemia at 3 months of age. Nine months after inoculation 15 of 45 inoculated had developed a generalized leukemia that was specific for (C3Hf × AKR)F_1 hybrids, *i.e.*, on transplant to other mouse strains the leukemia grew only in the F_1 hybrid. Each leukemia was tested for specificity by inoculating AKR, C3Hf, and (C3Hf × AKR)F_1 hybrids, and each grew progressively in the hybrids only, killing the mice in from 2 to 4 weeks. All surviving mice were kept under observation for 3 to 6 months. Representative tests are shown in table 4.

In the hybrid control group of 44 mice under observation, only 2 had developed leukemia at 9 months of age (table 1). The incidence of leukemia in these two groups in a 14-month period was 60 percent for the mice receiving the unheated filtrate and 23 percent for the control hybrids. Figure 1 represents a typical leukemia as seen in the inoculated hybrids.

TABLE 4.—*Specificity of the leukemias developing in the (C3Hf × AKR)F_1 hybrids inoculated when newborn with filtrates prepared from the tissue of leukemic AKR mice*

Source of leukemic tissue	Strain of mouse inoculated	Number inoculated	Number developing progressive leukemia	Time required to kill (*days*)
(C3Hf × AKR)F_1 leukemia at 3 months	C3Hf	2	0	—
	AKR	2	0	—
	(C3Hf × AKR)F_1	2	2	16
(C3Hf × AKR)F_1 leukemia at 4 months	C3Hf	2	0	—
	AKR	2	0	—
	(C3Hf × AKR)F_1	2	2	14
(C3Hf × AKR)F_1 leukemia at 4 months	C3Hf	2	0	—
	AKR	2	0	—
	(C3Hf × AKR)F_1	2	2	18

All mice had palpable and enlarged peripheral lymph nodes and spleen and at autopsy showed an enlarged thymus, an enlarged mesenteric node, and an enlarged liver. The involved organs often had hemorrhagic areas. Counts made of the peripheral blood of acutely ill mice generally showed an elevated white count, and blood smears showed the predominating cells to be abnormal cells of the lymphocyte group. These appeared to be larger than normal lymphocytes and had a round nucleus, which generally had a clear zone around it, and had one or more prominent nucleoli. Histologic sections made of the liver, spleen, lymph nodes, kidneys, and gonads showed heavy infiltration of these abnormal cells.

Leukemia-Producing Agent in (C3Hf × AKR)F_1 Hybrids

As the F_1 hybrids were sacrificed for tests on the specificity of the leukemia, part of the tissue was homogenized, extracted, and filtered as described under "Materials and Methods," and inoculated in newborn F_1 hybrids if available. At the time most of the mice were developing leukemia the mice were not breeding well, but a few experiments were carried out which again demonstrated the leukemogenic activity of leukemia cell-free filtrates. Curiously, however, in the first experiments only leukemias developed in the hybrid mice receiving filtrates from strain AKR leukemic mice, while in the group of hybrid mice receiving filtrates from the leukemic F_1 hybrids some developed leukemia and others parotid-gland tumors and, in some instances, adrenal-gland tumors, the neoplasms appearing at an early age. The results are given in table 5.

In these and the succeeding experiments, the C3Hf females used for breeding the hybrids were obtained from Dr. Jay's stock, whereas in the first series of experiments the C3Hf females were obtained from Dr. Law. Whether this change accounts for the difference observed has not been ascertained.

This second series of experiments is of special interest. In experiment 1, table 5, 11 newborn (C3Hf × AKR)F_1 hybrid mice—8 to 10 hours old—were inoculated with 0.1 ml. of filtrate prepared from a leukemia arising in an F_1 hybrid from the first series of experiments. Eight survived the inoculation; 3 died shortly afterwards with no lesions attributable to the inoculum; and the remaining 5 developed parotid-gland tumors in from 3 to 6 months. Of these 5, 2 also had mammary tumors and adrenal tumors. In both mice the mammary tumors appeared at 3 months and preceded the parotid tumors. It is rare for mammary tumors to develop at such an early age. Both mice were pregnant at the time the tumors developed. These mice were sacrificed when 4 months old, and both were found to have adrenal tumors of the same type as described previously (*5*), figure 2.

In experiment 2 (table 5), a litter of 6 newborn (C3Hf × AKR)F_1 hybrids less than 8 hours old was inoculated with 0.1 ml. of a filtrate prepared from transplanted AK-n leukemia #60 (this leukemia arose in an AK-n mouse obtained from Dr. L. Gross, Veterans Administration

TABLE 5.—*Leukemia and other tumors in (C3Hf × AKR)F_1 hybrid mice inoculated when newborn with filtrates from mouse leukemic tissues (second series)*

Expt. No.	Source of leukemic filtrate	Number of mice inoculated	Number surviving 3 weeks or longer	Number developing leukemia	Number developing parotid tumors	Number developing mammary tumors	Number developing adrenal tumors	Ages at which tumors appeared (*months*)
1	(C3Hf × AKR)F_1	11	5	0	5	2	2	3–6
2	AK-n #60 (G9)	6	5	5	0	0	0	2½–5
3	AKR #RIL 6 (G51)	7	4*	3	0	0	0	8–10
4	(C3Hf × AKR)F_1	14	11	5†	7	0	1	6–12
5	(C3Hf × AKR)F_1	7	3	2	0	0	0	6, 7
6	(C3Hf × AKR)F_1	7	7	2	0	0	0	9–12
7	(C3Hf × AKR)F_1	4	3	1	1	0	0	10
8	(C3Hf × AKR)F_1	7	5	5	0	0	0	4–7
9	AK-n #60 (G11)	7	3	1‡	3	1	2	3–4
10	(C3Hf × AKR)F_1	9	7	0	0	0	0	—
	Total	79	53	24	16	3	5	

*One female in this group when 9 months old was found to have bilateral ovarian tumors only.
†Two of these also had early parotid-gland tumors and one had an early adrenal tumor.
‡Mouse also had early parotid- and adrenal-gland tumors.

Hospital, Bronx, N. Y.). The tumor was removed from a mouse bearing the ninth transplant generation and was kept in a moist chamber at 4° C. for 48 hours before the filtrate was prepared from it. Five mice survived the inoculation and all developed leukemia in from 10 weeks to 5 months of age. In each instance transplants were made to test for specificity of the leukemia, as shown in table 4, and in each instance the leukemias were found to grow progressively only in the strain of origin or the (C3Hf $\times$ AKR)F_1 hybrid. In experiment 9 of this series (table 5), 7 newborn (C3Hf $\times$ AKR)F_1 mice 4 to 6 hours old were inoculated with a filtrate prepared from the tumor of a mouse bearing the eleventh transplant generation of AK-n leukemia #60. Four died within 2 weeks with no lesions attributable to the inoculum. Of the 3 surviving, 1 developed leukemia and 2 (one male and one female) developed parotid-gland tumors; the female also had a mammary tumor which appeared at 3 months of age (fig. 3), and when sacrificed at 4 months, it was found to have an adrenal tumor also. The male also had what appeared to be an early adrenal tumor. The medullary origin of these tumors is shown in figure 4.

Histologic sections of the organs of the mouse which developed leukemia in experiment 9 not only showed a heavy infiltration of leukemia cells but the parotid glands showed the typical nodular formation surrounded by hyaline material which has been seen in the parotid tumors, and one adrenal gland had what appeared to be an early medullary tumor. This mouse was sacrificed when 5 months old. These changes in the adrenal and parotid glands are shown in figures 5 and 6, respectively.

In these experiments in a 12-month period of observation, out of a total of 53 mice reaching maturity, 69.6 percent developed neoplasms—24 had leukemias, 16 had parotid-gland tumors, 3 of the group had mammary tumors, 5 had adrenal tumors in addition to the parotid tumors, and 1 female had bilateral ovarian tumors. Three of this group had both leukemia and parotid-gland tumors and one had early adrenal medullary changes.

Only 1 mouse in the control group of 33 (C3Hf $\times$ AKR)F_1 hybrids of this second series developed a neoplasm within the 9-month period. This neoplasm appeared in a male that was just 9 months old; the mouse had died of pneumonia but it had an enlarged thymus, which on microscopic examination appeared to be involved by a lymphoma. At 12 months, 15 percent had developed leukemia. No other tumors were observed in the control group.

Discussion

The relative resistance of strain C3H and C3Hf mice to the leukemogenic activity of cell-free filtrates prepared from the tissues of leukemic strain AKR mice and the high susceptibility of the (C3Hf $\times$ AKR)F_1 hybrid mice to this agent are similar to observations on the action of certain viruses on different strains of one species. For example, the relation of different bacterial viruses to their host provides some of the best examples of biologic specificity known. Burnet *et al.* (*6*) in 1937 showed that

viruses are frequently capable of distinguishing sharply between bacterial strains indistinguishable antigenically.

Although the leukemia-producing agent demonstrated in cell-free filtrates prepared from strain AKR mouse leukemic tissues has not been shown to be a virus, it does appear to have a biologic specificity similar to that exhibited by certain viruses. Only the hybrid mice genetically related to the AKR mice showed a high degree of susceptibility to the agent.

As was found in strains C3H and C3Hf mice (*5*), the (C3Hf × AKR)F_1 hybrid mice also showed susceptibility to early tumor formation as a result of the inoculation of the newborn mice with filtrates prepared from mouse leukemic tissues; four, and possibly five, neoplasms were produced in mice 2.5 to 9 months old. In the mice inoculated, the neoplasms occurred in the following frequency: lymphatic leukemias, parotid-gland tumors, adrenal-gland tumors, mammary tumors, and ovarian tumors.[3] Many of the mice bearing 2 or 3 tumors were only 3 months old—unusually young for any tumor to occur. Mammary tumors produced in mice susceptible to the mammary tumor milk agent occur 6 to 8 months after the inoculation of suckling mice with the agent. Attempts at decreasing the interval between the time of administering the agent and the appearance of the tumor have been unsuccessful. A series of experiments is in progress to determine if early mammary tumors can be induced by inoculating newborn mice (rather than the older mice) with cell-free extracts prepared from a transplanted mammary tumor which arose in one of the experimental hybrids and from extracts prepared from mammary tumors carrying the milk agent.

The low susceptibility of strain C3H mice to a leukemia-producing agent, as observed in these and previous experiments, is not in agreement with Gross's observations (*7–11*). At present the reason for this difference is not apparent.

Summary

1) A leukemia-producing agent was demonstrated in tissues of leukemic AKR mice by inoculating newborn (C3Hf × AKR)F_1 hybrid mice with cell-free filtrates of the tissues.

2) The incidence of leukemia in one group of inoculated F_1 hybrids in a 14-month observation period was 60 percent, as compared with 23 percent in control animals. In a second series, neoplasms were observed in 70 percent of inoculated F_1 hybrids in a 12-month observation period, of which 45 percent were leukemias, 30 percent were parotid tumors and 9 percent were adrenal tumors. Six percent had both parotid- and adrenal-gland tumors.

3) A series of C3H and C3Hf mice inoculated with the same filtrates as used in the F_1 hybrids remained free of leukemia, with the exception of 1 mouse that developed leukemia when 14 months old.

[3] Only 1 mouse of the group had ovarian tumors; these occurred bilaterally. Two mice of strain C3H were observed to have the same type of ovarian tumor (*5*). The relation of these tumors to the experimental procedure is not known at this time.

4) The leukemias appeared much earlier in the inoculated mice than in the controls, appearing as early as 10 to 12 weeks after inoculation of the newborn mice.

5) In all instances, when transplanted to other mice the leukemias grew progressively only in (C3Hf × AKR)F_1 hybrids or the strain of origin.

6) A leukemia-producing agent was demonstrated in leukemias arising in the F_1 hybrids. Tumors of the parotid gland and adrenal glands were also observed in F_1 hybrids and apparently resulted from inoculation with filtrates from the F_1 leukemias.

7) Mammary tumors were observed in two of the inoculated (C3Hf × AKR)F_1 hybrids 3 months old.

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PLATE 1

FIGURE 1.—(C3Hf × AKR)F_1 hybrid male, 4 months old, inoculated when newborn with filtrate of leukemic AKR mouse tissue. Generalized leukemia. Note enlargement of peripheral lymph nodes, mesenteric node, thymus, liver, and spleen. × 1.5

FIGURE 2.—(C3Hf × AKR)F_1 hybrid female, 4 months old, inoculated when newborn with filtrate of leukemic F_1 hybrid mouse tissue. Bilateral tumors of the parotid gland. Mammary tumor observed when mouse was 3 months old. *Left* adrenal tumor. Normal size.

FIGURE 3.—Mammary tumor from above mouse showing typical morphology of mammary tumor. Hematoxylin and eosin. × 350

FIGURE 4.—(C3Hf × AKR)F_1 hybrid male, 4 months old, inoculated when newborn with filtrate of tissues of a (C3Hf × AKR)F_1 mouse with a generalized leukemia (expt. 1, table 5). Adrenal tumor. Tumor tissue replaces part of the medulla, and compresses cortex—*lower left*. Hematoxylin and eosin. × 73

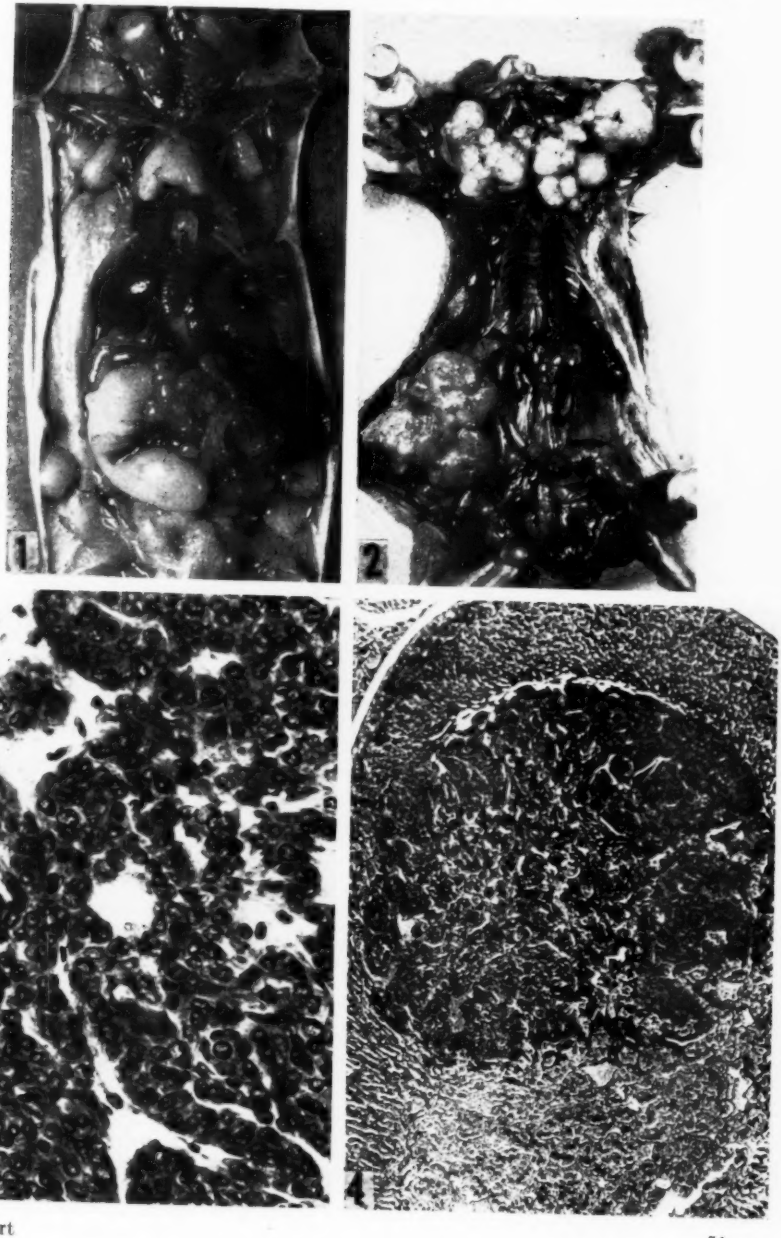

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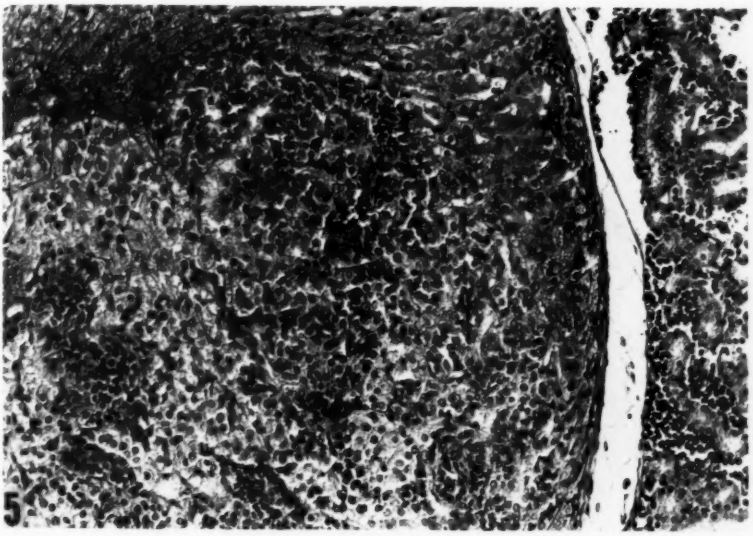
PLATE 2

FIGURE 5.—(C3Hf × AKR)F_1 hybrid male, 5 months old, inoculated when newborn with filtrate of tissues of an AKR mouse bearing the 11th transplant generation of leukemia #60. Adrenal gland. Groups of cells of medulla on *lower left* and *upper right* are more basophilic and may represent early neoplastic change. This mouse had leukemia; infiltration of leukemic cells into kidney cortex is shown. Hematoxylin and eosin. × 200

FIGURE 6.—Same mouse as in figure 5. Parotid gland. Groups of acinar cells are separated by masses of eosinophilic hyaline substance (*arrow*). This is frequently seen in these neoplasms of the parotid gland. Tissue in center of photograph appears normal. Hematoxylin and eosin. × 143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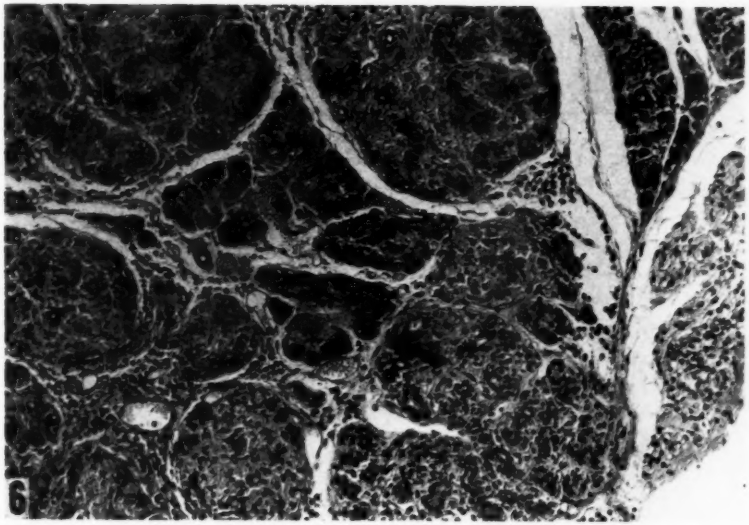
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Experimental Studies in Metal Cancerigenesis. IV. Cancer Produced by Parenterally Introduced Metallic Nickel[1]

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The rapidly expanding production and use of metallic nickel and its compounds in the chemical, metallurgical, aviation, and other industries (paint, ink, linoleum, oil, etc.), as well as the increasing introduction of some of their products into the home in the form of kitchen utensils, household goods, paints, and other consumer goods, provide extensive contacts of large population groups with this metal. The significance of metallic nickel as a carcinogen so far has been restricted to the occurrence of cancers of the nasal cavity, paranasal sinuses, and lung among English, Norwegian, and Canadian nickel refinery workers (*1*) and to the experimental production of cancers in rats at the sites of implanted nickel powder (*2*).

Because of the limited scope of the initial experimental studies, a reinvestigation of the problem on an enlarged and modified scale was undertaken. In the original experiment previously reported, the following were used: rats of the Osborne-Mendel strain, nickel obtained from the Chicago Apparatus Company, and lanolin as the dispersing vehicle. In the present study nickel provided through the courtesy of the International Nickel Company was employed. In addition to rats of the Osborne-Mendel strain, animals of the Wistar strain were used so as to eliminate, or at least mitigate, any special strain-specific influence upon the type of reactions elicited. For overcoming any possible effects of species-specific factors, other animals, *viz.*, mice, guinea pigs, and rabbits, were given nickel by various routes (intrafemoral, intramuscular, intravenous, intraperitoneal, intrapleural). A highly viscous gelatin solution was substituted for lanolin in most of these experiments in view of the alleged anticarcinogenic action of this material, reported in connection with the testing of carcinogenic polycyclic hydrocarbons (*3–5*).

Experimental Procedures

The powdered metallic nickel purchased from the Chicago Apparatus Company consisted of irregularly shaped, ragged particles ranging in

[1] Received for publication December 15, 1954.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

diameter from 2 to 50 microns with the mean between 10 to 30 microns. The powdered nickel obtained from the International Nickel Company, on the other hand, was prepared by the disintegration of nickel carbonyl and was composed of minute round particles usually clumped together in small chains or in loose, larger clumps. The individual particles as a rule measured less than 1 micron in diameter, although the average-sized clump or branched chain of beads was usually 2 to 20 microns in length, with some clumps as large as 100 microns. While the powdered metal in its original form was employed in all implantation experiments, a fraction of nickel (International Nickel Company) consisting of particles measuring 0 to 4 microns in diameter was prepared through fractionation of the powder in a particle analyzer (American Instrument Company) for intravenous injection.

I. Intrafemoral Implantation of Nickel

Rats, rabbits, and dogs were used for implanting a suspension of powdered nickel in the marrow cavity of the femur. Since the dogs that were operated on more than 5 years ago have so far not shown any gross evidence of tumor formation and since they will be kept under observation for an additional 2 to 3 years, no further reference will be made to this aspect of the experiment.

a) Two series of 50 rats each of the Wistar strain, approximately 3 months old and equally divided as to sex, were used for implanting about 50 mg. of powdered nickel obtained from the International Nickel Company (INCO) and the Chicago Apparatus Company (CAC), respectively. The rats were anesthetized for this purpose. A hole was drilled through the cortex of the right femur into the marrow cavity with an electric hobby drill having a flexible shaft and provided with a #1 dental burr. Then by use of a tuberculin syringe and a 20-gauge needle, about 0.1 cc. of a 5-percent nickel suspension in a 20-percent gelatin solution prepared with physiologic saline solution was injected through this hole into the marrow cavity.

Through back pressure there was usually some seepage of the suspension from the marrow cavity around the needle into the periosteal tissue. The great majority of the rats tolerated the surgical intervention well. A few rats later developed abscesses and osteomyelitic complications, sometimes associated with fistula formation. Differential leukocyte counts were made before the injection of nickel and again about 12 months later. No significant changes were noted except for a rise in eosinophilic leukocytes from an average of 2 percent before the injection to an average of 4.1 percent in the rats after implantation with INCO nickel, and a rise from 2.3 percent to 9.1 percent in rats given CAC nickel.

Following an observation period of 18 months, during which time 9 tumors of the right thigh were found among the rats of the CAC series, the 20 surviving animals were reinjected in the left femur with the same dose of nickel previously used for the right thigh. This experiment was performed for testing whether the first deposition of nickel and a possible

systemic immunochemical reaction to it might influence the relative tendency toward tumor formation in a positive or negative direction in the as yet untreated left thigh. During the subsequent 10 months of the total survival period of 28 months (at the end of which time the last surviving rats were sacrificed), one tumor developed in the left thigh, while three appeared in the right thigh (table 1).

The first tumors at the site of nickel implantation appeared after a minimal induction period of 4 to 5 months. A total of 27 cancers appeared among the 100 rats composing the two groups. The relative incidence rate of tumor formation for the two types of nickel used was practically identical. The tumors varied in size from 1 cm. to 6 cm. in diameter. They were usually of firm consistency, attached to the femur, sometimes extending to and involving the pelvic girdle, and they were of medullary whitish-pink appearance and fibrillar texture on the cut surface. The centers of the larger tumors as a rule were soft, dirty grayish-red, and

TABLE 1.—*Survival periods and tumor formation in thighs of rats after intrafemoral deposition of nickel*

Months.....	1–3	4–6	7–9	10–12	13–15	16–18	19–24	25–28	Total
Deaths INCO.....	1	9	2	3	3	12	11	9	50
Deaths CAC.....	.....	5	7	5	3	10	10	10	50
Tumors INCO.....	.....	5	.....	2	.....	1	5	1	14
Tumors CAC.....	.....	1	5	1	.....	2	3	1	13

necrotic. In several tumors the neoplastic tissue surrounded black deposits. Sixteen of these neoplasms were present in female rats and 11 in males.

The histologic examination of these cancers showed them to be of different histogenetic origin. The majority (16) consisted of large, round, oval, spindle, or elongated cells with intercellular fibril formation indicating their origin from periosteal connective tissue. However, 4 of these cancers had histologic characteristics of muscular derivation; 5 displayed cellular whirl formation and nuclear garland together with a palisade arrangement suggesting a neurogenic origin; 1 exhibited an angiosarcomatous structure; and 1 had histologic features suggesting its reticulum-cell derivation from bone-marrow cells (figs. 1 and 2). Metastases were noted in 14 of these tumor rats (9 INCO, 5 CAC). They involved regional and remote lymph nodes (abdominal and mediastinal), kidney, spleen, pancreas, liver, lung, and heart (fig. 3).

In addition to these malignant tumors at the site of nickel deposition, a considerable number of additional malignant and benign neoplasms were observed among the rats of both series. Some of them occurred in rats with nickel sarcomas; others were found in animals without such cancerous reactions in the thigh. Several animals had multiple primary tumors. Two rats had round-cell sarcomas of the ileocecal lymph nodes extending into the retroperitoneal and mesenteric lymph nodes. Four rats had single or multiple adenofibromas of the breast, while a fifth

showed an adenocarcinoma of the mammary gland. In three of these rats with breast tumors, there was also present a hemangioma of the adrenal medulla, and in one rat there was a basophilic adenoma of the anterior lobe of the hypophysis. Adrenal hemangiomas were noted in 2 additional rats. Basophilic adenomas of the pituitary gland occurred in four additional rats. One rat had a myeloid leukemia; another, a cystadenoma of the ovary. A pancreatic insuloma measuring 2 mm. in diameter was discovered in one rat during the histologic examination of the sections (fig. 4). Altogether there were 20 additional tumors present in the animals of both series, making a total of 47 malignant and benign neoplasms among the 100 rats used.

Control rats intrafemorally injected with gelatin.—Twenty-three female rats of the Wistar strain, 7 months old, were injected through a drill hole into the marrow cavity of the right femur with 0.1 cc. of a 20-percent gelatin solution prepared with physiologic saline. The surgical procedure and the injection of gelatin were tolerated without untoward effects. Table 2 gives the survival periods of these rats.

TABLE 2.—*Survival periods of rats intrafemorally injected with gelatin solution*

Months	4–6	7–9	10–12	13–15	16–18	19–21	22–24
Deaths	2	3	1	0	2	5	10

None of the rats developed a tumor of the right thigh. There were, however, 10 benign or malignant neoplasms among these rats. Multiple tumors of the same type were present in two different rats and multiple tumors of different types were present in 4 rats. Two rats had round-cell sarcomas of the ileocecal lymph nodes forming round masses measuring up to 10 cm. in diameter. A third rat had a nodular, soft, multicystic, fused mass in this region, which upon histologic examination revealed a lymphangiectatic, edematous structure with solid cellular areas composed of small and large mononuclear cells, suggesting a presarcomatous condition. A fourth rat displayed at necropsy a solitary, firm node measuring 3 cm. in diameter and located in the ileocecal region with several smaller ones at the mesenteric root. The cut surface of the large node was medullary and white and showed a cheesy, yellowish center. The wall of the large node was composed of a cellular connective tissue which formed the wall of a cyst lined by squamous-cell carcinomatous masses. These masses infiltrated the wall in trabeculae composed of polygonal and spindle-shaped epithelial cells with hyperchromatic nuclei. Five rats had adenomas or adenofibromas of the breast which were multiple in two of them and which measured up to 10 cm. in diameter. In one rat the breast tumor was associated with a basophilic pituitary adenoma. There were three additional rats with basophilic pituitary adenomas and one rat with an adrenal hemangioma. The largest of both types of these tumors measured 1 cm. in diameter and were dark red in

color. One rat had an extensive, and in part, nodular lymphoid leukemia (involving the liver, spleen, kidneys, adrenals, heart, lung, and brain) causing a considerable enlargement of the liver and spleen which measured 8 × 2.5 × 1 cm. There were thus 14 tumors present in the 23 control rats.

Comment.—From the observations reported, it may be concluded that the 27 sarcomas of different histogenetic origin found at the site of nickel deposition are causally related to the metal and thus represent true metal cancers. These findings on the cancerigenic action of nickel in rats confirm those previously reported, when 6 sarcomas of different types and 1 squamous-cell carcinoma were found in 25 rats intrafemorally injected with a similar amount of nickel (CAC) suspended in lanolin. In both experiments the total nickel cancer yield was about 28 percent of the total number of rats injected (125). Regional and distant metastases were found in 17 of the 34 cancers. The total absence of cancers of the thigh in the rats injected with gelatin only indicates the specificity of the nickel effect. The wide variation in histogenetic types of cancer observed in these rats, ranging from osteogenic sarcoma through rhabdomyosarcoma, neurogenic sarcoma, angiosarcoma, and reticulum-cell sarcoma to squamous-cell carcinoma, indicates that no tissue specificity exists in relation to the cancerigenic action of nickel.

The presence of a considerable number and variety of benign and malignant tumors remote from the site of nickel deposition (25 tumors among 125 rats) raises the question as to whether these may be attributable to a systemic action of nickel producing a spectrum of different tumors. However, a comparison of these neoplastic reactions with those noted among the rats comprising the two control groups (gelatin and lanolin) (*6*), which exhibited a total of 17 different tumors among 48 rats, and with those found in rats intrafemorally injected with beryllium, arsenic, and asbestos does not support such a view, especially as these neoplasms were similar in histologic type and topographic distribution to those seen in the test series. The conclusion seems to be justified that the tumors which developed at a site remote from the site of nickel implantation were not causally related to nickel.

There is no evidence that the creation of a nickel depot influenced in any distinct way the subsequent tumor related to the production of a second depot made at another site some 18 months later. Although the only sarcoma which developed in the left thigh appeared as early as 4 months after the second nickel implant was placed and exhibited an explosive growth, no other tumor development in the left thigh followed. Moreover, once the tumor had become established, an extraordinarily high proliferative rate was observed with many other nickel cancers.

b) Six 3-month-old Dutch rabbits weighing between 1,750 and 2,000 gm. were injected into the right femoral cavity with about 0.25 cc. of powdered nickel (CAC) suspended in lanolin (12.5-percent nickel by volume, 43.8-percent by weight). A second injection was given 27 months later, when twice the original dose was administered. The

rabbits recovered well from these procedures and gained weight, reaching a maximal weight between 3,000 and 4,000 gm. Blood studies made at varying intervals up to 24 months after the initial injection of nickel did not reveal any significant changes in the number of erythrocytes and leukocytes, in the differential count and in the amount of hemoglobin.

Two additional rabbits were injected with lanolin only.

The necropsy and histologic observations made on 4 of the 6 rabbits injected with nickel revealed the presence of a completely hyalinized spleen with highly atrophic lymph follicles and a hyalinosis of the renal glomeruli in one of them. In a second rabbit there was a nodular mass measuring 4 cm. in diameter located at the junction of the uterine horns. Upon dissection, a soft, reddish mass of homogeneous texture was found projecting into the distended uterine lumen which contained a moderate amount of serous fluid. The node consisted of closely packed, papillary glandular formations often forming cysts lined by a papillary columnar epithelium. Since there was no invasion of the myometrium by these glandular structures, the node represented an endometrial adenoma.

The sixth rabbit, which died 43 months after the implantation of nickel, showed at necropsy a mass of well encapsulated, firm nodules measuring $3 \times 3 \times 3$ cm. to $5 \times 3 \times 3$ cm. in diameter located in the right thigh. The centers of these nodes were hemorrhagic, while the marginal portions were grayish-pink in color and homogeneous in texture. Numerous similar medullary nodules were present in both kidneys, spleen, right ovary, parametrium, liver, cervical lymph nodes, and lungs. The largest of these nodules measured 4 cm. in diameter. Survival periods for these rabbits are presented in table 3.

TABLE 3.—*Survival periods of rabbits injected intrafemorally with nickel (CAC)*

Months	13–18	31–36	37–42	43–48
Deaths	1	1	3	1

The histologic examination showed the nodular masses to be composed of irregularly arranged bundles of slender spindle cells surrounded by a moderate amount of fibrillar connective tissue diffusely invading the surrounding normal tissues. The examination of the right femur revealed the presence of similar tumor growth within the marrow cavity (figs. 5 and 6). In view of the location of the tumor at the site of injection of the nickel, it may be concluded that the fibrosarcoma represents a cancerous response to the nickel. The observation indicates that the cancerigenic action of nickel is not restricted to rats but extends to other species.

II. Intravenous Injection of Nickel

Mice, rats, and rabbits were injected with a suspension of powdered nickel (INCO) in gelatin solution prepared with physiologic saline.

a) Twenty-five strain C57BL male mice, 6 weeks of age, were injected in the tail vein with 0.05 cc. of an 0.005-percent suspension of nickel powder in a 2.5-percent gelatin solution. The injections were repeated after a 2-week interval. Four mice died immediately after the second injection.

There were no pertinent gross and histologic observations, except for circulatory disturbances in some of these animals. Table 4 gives the survival periods of the mice of this group.

TABLE 4.—*Survival periods of mice after intravenous injections of nickel*

Months	0–1	5–15	13–14	15–16
Deaths	4	2	13	6

b) Twenty-five 3-month-old rats of the Wistar strain were injected in the vena saphena with an 0.5-percent nickel suspension prepared with physiologic saline. The nickel powder used was composed of particles measuring 0 to 4 microns in diameter. The individual dose given was 0.5 cc. per kg. of body weight (0.1–0.18 cc. per rat ranging in weight from 100–184 gm.). A total of six injections was given at one-week intervals.

Four rats died during the injection. Differential leukocyte counts made before the first injection and on two occasions during the last 8 months following injection did not reveal any significant changes. The last surviving rats were sacrificed at the end of an observation period of 28 months (table 5).

TABLE 5.—*Survival periods of rats after repeated intravenous injections of nickel*

Months	0–3	4–6	7–9	10–12	13–18	19–24	25–28
Deaths	5	3	10	1	0	3	3
Tumors	0	0	7	0	0	0	0

Seven rats developed tumors in the region of the groin and in the proximal portion of the thigh, where nickel had escaped through leakage from the vena saphena into the subcutaneous tissue. They appeared about 7 to 8 months after the first injection. The tumors were bilateral in one rat and measured from 1 to 5 cm. in diameter. They were firm and encapsulated toward the skin but adherent to the pelvic girdle or femur. Metastases were formed by only one of these tumors which produced a fused, nodular retroperitoneal mass measuring $2 \times 4 \times 0.5$ cm., and several hemorrhagic nodules in the lungs measuring up to 1 cm. in diameter.

Upon histologic examination, these tumors were found to consist of masses of large spindle cells, which in two of them were apparently of myoblastic character, while in one they were associated with trabecular bone incorporated into the spindle-cell matrix. Masses of multinucleated, huge giant cells representing apparently regenerating muscle cells were present within the tumor masses of several rats. In another rat, the

histologic examination of the tissue of the groin revealed a highly cellular, fibroblastic tissue which, through its cellular irregularity, suggested a presarcomatous condition (figs. 7 and 8).

In addition to these nickel-induced tumors there were other neoplasms remote from the site of nickel deposition in some of the rats (1 fibroadenoma of the breast, 2 adenomas of the breast with atypical glandular, papillary and solid epithelial formations, 3 basophilic pituitary adenomas measuring up to 1 cm. in diameter, and 3 hemangiomas of the adrenal medulla, 2 of which were associated with the hypophyseal tumors).

The lungs of 2 rats showed emboli of black, amorphous material which was evidently nickel powder. Similar deposits were not observed in the reticuloendothelial cells of the various organs studied.

c) Ten albino rabbits, about 3 months old, weighing from 2,000 to 3,000 gm., were given at weekly intervals six injections of a 1-percent powdered nickel suspension (0.5 cc. per kg. of body weight) in 25-percent gelatin solution. Five control rabbits received by vein similar amounts of the 25-percent gelatin solution.

The injections were not well tolerated. The rabbits had convulsive reactions developing during the injection of the dispersion. Some of the nickel leaked from the vein into the surrounding tissue where it gave rise to black induration, necroses, and abscesses. Several of the rabbits gained some weight during the period of the injections; others lost a few hundred grams. Two rabbits died with paresis of the hind legs 4 and 12 months, respectively, after the start of the experiment. The others died or were sacrificed at various times during the experiment. The control rabbits were healthy 40 months after the start of the experiment (table 6).

TABLE 6.—*Survival periods of rabbits after repeated intravenous nickel injections*

Months	0–12	13–18	19–24	25–32	33–36	37–44
Deaths	4	1	1	3	1	Test series

Erythrocyte, leukocyte and differential counts, determinations of the coagulation and bleeding times, and of the hemoglobin content were done at varying intervals and the urine was examined for albumin, sugar, acetone, blood, bile, and the presence of casts and cells in the sediment.

There were no significant changes in these elements apart from a 50- to 100-percent increase in bleeding time after the sixth injection. Since this phenomenon was common to both the test and the control series, it is apparently elicited by the introduction of the macromolecular gelatin molecules into the blood (*7*).

The necropsy findings in all rabbits were negative for tumors. In the pulmonary capillaries of two rabbits, black amorphous clumps were present which were surrounded by multinucleated endothelial syncytial formations. One rabbit had a completely hyaline pulp with atrophic lymph follicles, as well as a marked interstitial fibrosis and parenchymal atrophy of the pancreas.

Comment: Repeated intravenous injections of nickel suspensions into mice, rats, and rabbits did not cause the development of tumors in any organ or tissue to which nickel particles might have been brought by the blood stream. The only cancers which were found originated in the subcutaneous tissue of rats into which nickel had seeped from leakage of the injected vein.

III. Intramuscular Injection of Nickel

Fifty strain C57BL mice, 6 weeks old, were injected in the muscle tissue of the right thigh with approximately 0.02 cc. of an 0.05-percent suspension of powdered nickel (INCO) in a 2.5-percent gelatin solution. Nine died during the first 9 months, 15 during the next 3 months, and 26 died or were sacrificed during the 13th to 18th month of the experiment. There were no local reactions at the site of deposition of the nickel. A few of the mice showed hyaline lesions in the spleen and liver. None developed a tumor at the site of injection. This negative experience in mice contrasts sharply with the positive observations made in rats, in which the seepage of nickel suspension from the femoral cavity into the surrounding connective tissue and muscle caused the development of fibrosarcomas and myosarcomas.

IV. Intrapleural Injection of Nickel

Fifty strain C57BL male mice, 6 weeks old, were injected in the right pleural cavity with 0.02 cc. of an 0.06-percent suspension of powdered nickel (INCO) in a 2.5-percent gelatin-saline solution. Table 7 gives the survival periods for these mice.

TABLE 7.—*Survival periods of mice intrapleurally injected with nickel*

Months	0–6	7–12	13–18	19–24
Deaths	8	5	33	4

The post-mortem examination and a histologic study of the organs of these mice showed enlarged spleens, kidneys, and livers with mild to marked hyaline lesions in some of the mice. There were no tumors.

The negative outcome of this experiment on mice is in agreement with the negative results obtained in the other studies in which this species was used. It contrasts, on the other hand, with the positive results previously obtained in rats, in which the intrapleural introduction of powdered nickel (CAC) suspended in lanolin was followed by the development of spindle-cell sarcomas of the thoracic wall near or around nickel deposits in 8 of 25 Osborne-Mendel rats.

Comments

The production of sarcomas at the sites of deposition of finely powdered metallic nickel (thigh and groin) in 28 percent of rats injected in the femoral cavity and/or saphenous vein with this metal suspended in gelatin

solution confirms previously reported observations concerning the development of cancers in the thigh and thoracic wall of rats following an introduction of nickel suspended in lanolin. Since tumor formation was noted in two different strains of rats (Osborne-Mendel and Wistar), with two different types of nickel (International Nickel Company and Chicago Apparatus Company) and with two different types of vehicle (lanolin and gelatin), there can be no doubt that nickel, when present in rather concentrated amounts in the tissue of rats, is capable of eliciting malignant tumors. The lack of a systemic cancerigenic action of nickel when generally dispersed through the blood after its intravenous administration suggests that either a minimal amount of this metal is needed at any one point for insuring a cancerigenic effect, or the body is able to dispose effectively of minute amounts of this metal when it is present in the form of very small particles.

Furthermore, the observations made indicate that the cancerigenic action is to a certain extent species-specific, since cancers were not obtained in mice intramuscularly, intrapleurally, or intravenously injected with nickel suspended in gelatin solution. The occurrence of a fibrosarcoma in the femur of a rabbit 43 months after an intrafemoral introduction of nickel in lanolin, on the other hand, demonstrates that rabbits apparently are susceptible to a cancerigenic action of nickel. The absence of similar reactions in rabbits intravenously injected with nickel may be explained by the fact that they did not survive sufficiently long to cover the induction period of metal cancers in rabbits, which seems to be in the neighborhood of 2 to 5 years (*8*).

Moreover, these positive observations made in two species provide additional evidence in support of the contention that the carcinogenic action in rats is a specific one and not an expression of some hypothetical nonspecific irritative effect of the metal seen only in rats (*6*).

The appreciable number of benign and malignant tumors found in rats remote from the deposition of nickel raises the question of a possible systemic spectrum effect of this metal apparently found in the form of lung cancers in rabbits following an intrafemoral implantation of chromium and cobalt (*8*), or resembling that effect noted with several carcinogenic amino compounds (4-aminodiphenyl, *p*-dimethylaminoazobenzene, and 2-acetylaminofluorene) in rats and dogs by several investigators (*9–11*). However, when comparing the number, types, and distribution of the various tumors found remote from the site of nickel introduction with those observed in rats belonging to the control series, it becomes most doubtful that such an action of nickel was present.

Special consideration in this respect, however, may be in order concerning the insuloma seen in one of the rats following an intrafemoral nickel implantation, since in another experiment three identical pancreatic tumors were found among 25 rats intraperitoneally or intrafemorally injected with another cancerigenic metal, namely, chromium. Because of the great rarity of these tumors not only in man but also especially in experimental animals (*12, 13*), the suspicion remains that the genesis of

these neoplasms may somehow be related to the administration of the two cancerigenic metals, although no information exists as to any special metabolic or toxic action of these metals upon the pancreatic islets similar to that exerted by alloxan, suggesting thereby a specific affinity of the metals for the cellular constituents of these endocrinic formations. There was also no anatomic evidence in the rat carrying the insuloma of an islet growth-stimulating action of estrogen or progesterone, recently reported for rats by Haist (*14*). Since there was no evidence of a glycogenesis of the liver in this particular rat, it is highly doubtful whether the tumor produced insulin.

According to the experimental evidence available, cancer formation has so far been observed after the implantation, intravenous injection, or inhalation of the following metals:

a) Nickel implanted intrafemorally, intrapleurally, and subcutaneously into rats and rabbits causing local tumors (*2*, *6*).

b) Chromium implanted intrafemorally into rabbits causing local and remote lung tumors (*8*).

c) Cobalt implanted intrafemorally into rabbits causing local and remote lung tumors (*8*) and implanted intramuscularly into rats causing local tumors (*15*).

d) Beryllium injected intravenously into rabbits eliciting sarcomas of bones [Gardner (*16*); Barnes (*17*); Barnes, Denz, and Sissons (*18*); Dutra, Largent, and Roth (*19*)]; and inhaled by rats producing bronchiogenic carcinomas (*20*).

e) Uranium implanted intrafemorally and intrapleurally into rats eliciting local cancers (*21*).

While some information on the biologic activity of chromium and cobalt exists, the data available on the biologic activity of nickel are very scanty. Nickel apparently forms complexes with proteins since cutaneous contact with this metal is not infrequently the cause of an allergenic dermatitis. It is prone to form chelates with organic compounds, such as dimethyl glyoxime, and may combine with carbamates and fatty acids. Chemical and biologic evidence indicates that it is an oxidation antagonist and a hydrogenation catalyst and may become soluble in tissue fluids. Recent spectrographic studies of Araki and Mure (*22*) have demonstrated its presence in benign and malignant tumors of man and experimental animals. The significance of nickel in tumors is not known. Its distribution in the various tissues resembles that of zinc. Since some students of nickel have suggested that it has a relation to insulin, Araki and Mure proposed that nickel may have an inhibitory action on tumor growth. It is evident from the enumeration of the various data available on the possible and actual biologic action mechanisms of nickel that its carcinogenic effect may take various biologic pathways, none of which so far has been explored.

It may be noteworthy in this connection that Wase, Goss, and Boyd (*23*) working with radioactive nickel, found that nickel apparently has a high value for the complex formation constant of nickel with lung protein.

The reasons for this affinity of nickel for lung protein, however, are as yet not known.

Conclusions

1) The intrafemoral and subcutaneous introduction of powdered nickel into rats resulted in the production of sarcomas of different histogenesis in about 28 percent of the implanted animals.

2) Of six rabbits that survived a similar procedure for over 3 years, one also developed a metastasizing endosteal fibrosarcoma of the femur. No tumors, on the other hand, were elicited in mice given nickel intravenously, intraperitoneally, or intramuscularly.

3) Although many benign and malignant tumors were found in rats remote from the site of nickel deposition, there is no reliable evidence indicating a carcinogenic spectrum effect of nickel similar to that reported by Schinz and Uehlinger for chromium and cobalt.

4) The occurrence of an insuloma in the pancreas of a rat intrafemorally implanted with nickel is reported.

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PLATE 3

FIGURE 1.—Neurogenic sarcoma in the thigh of a rat with intrafemorally implanted nickel showing palisade and garland arrangement of nuclei. × 340

FIGURE 2.—Myoblastic sarcoma in the thigh of a rat with intrafemorally implanted nickel showing multinucleated myoblastic cells. × 340

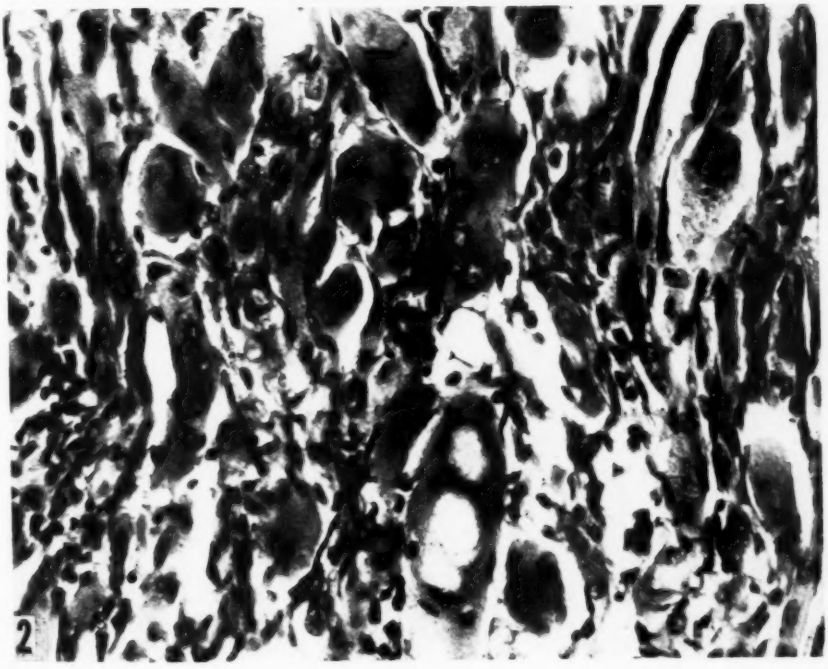

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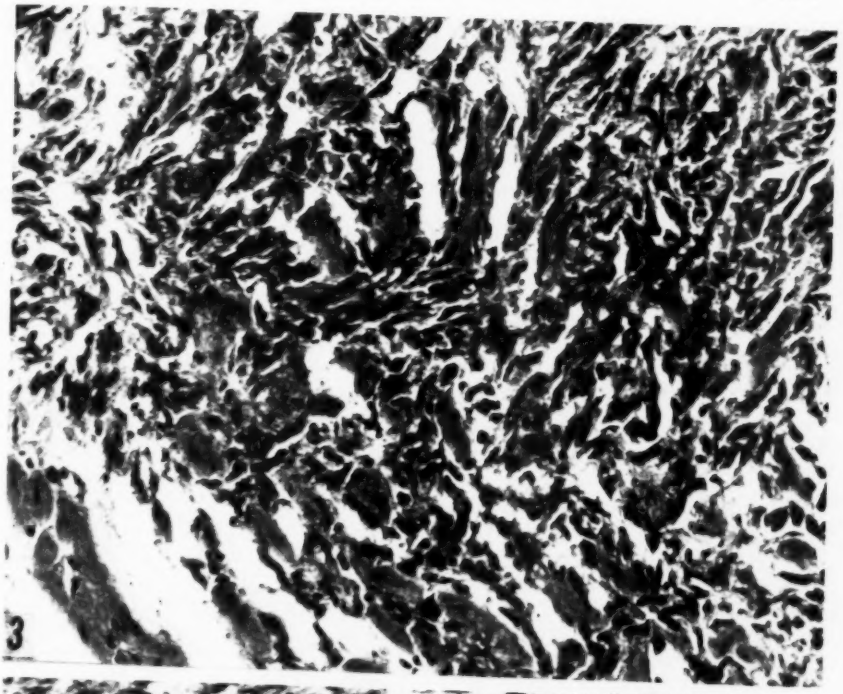
PLATE 4

FIGURE 3.—Spindle-cell sarcoma in the heart of a rat following intrafemoral implantation of nickel. × 340

FIGURE 4.—Endosteal fibrosarcoma of the narrow cavity of the femur of a rabbit following intrafemoral implantation of nickel. × 340



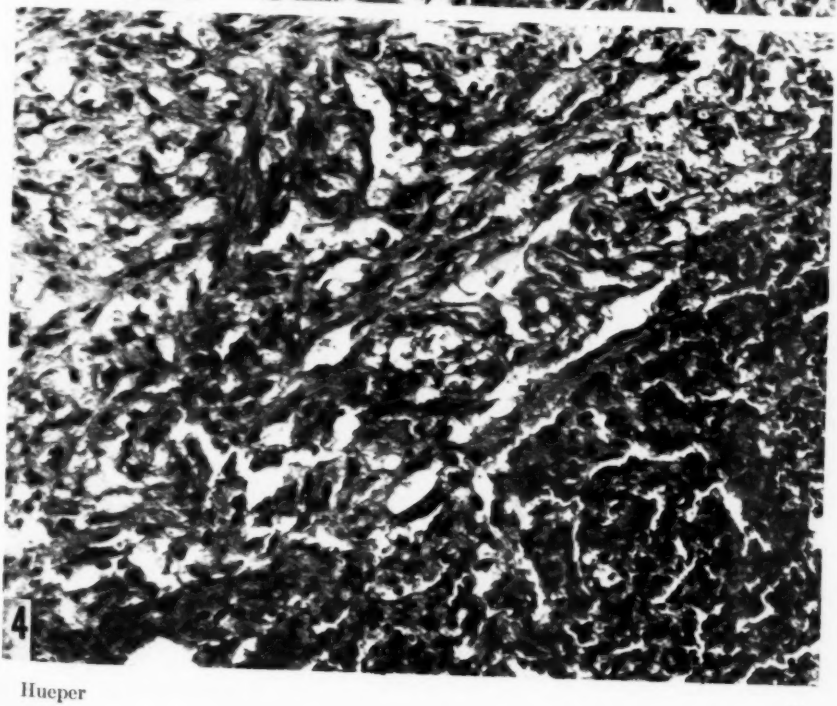


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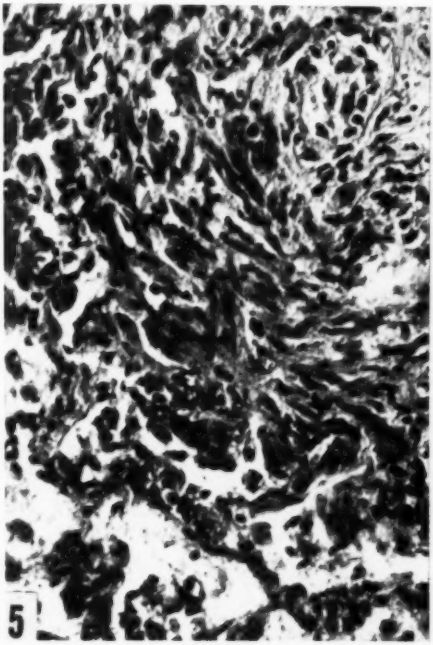
PLATE 5

FIGURE 5.—Metastatic fibrosarcoma in the lung of a rabbit from an endosteal primary cancer. × 340

FIGURE 6.—Insuloma in the pancreas of a rat intrafemorally injected with nickel. × 43

FIGURE 7.—Metastatic oval-cell sarcoma in the kidney of a rat originating from a primary sarcoma of the groin following injection of nickel into the vena saphena. × 340

FIGURE 8.—Metastatic spindle-cell sarcoma in the lung of a rat following intravenous injection of nickel into the vena saphena and the formation of a sarcoma in the region of the groin. × 340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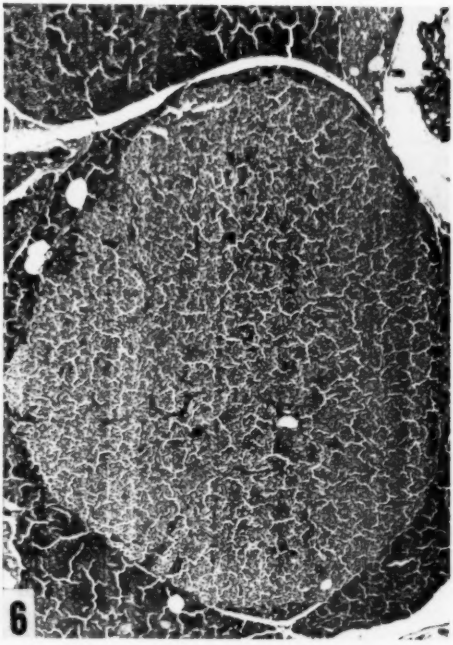
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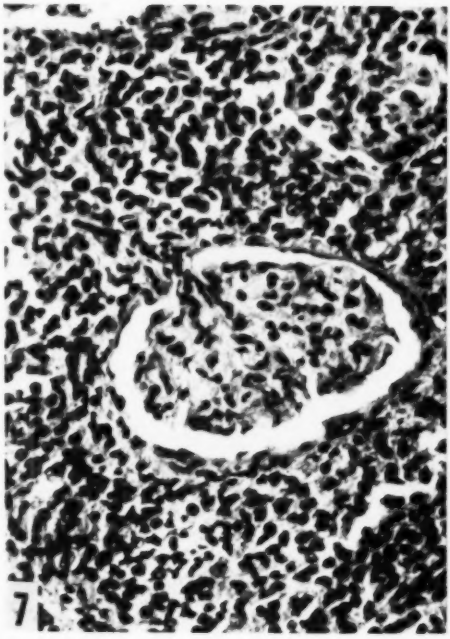
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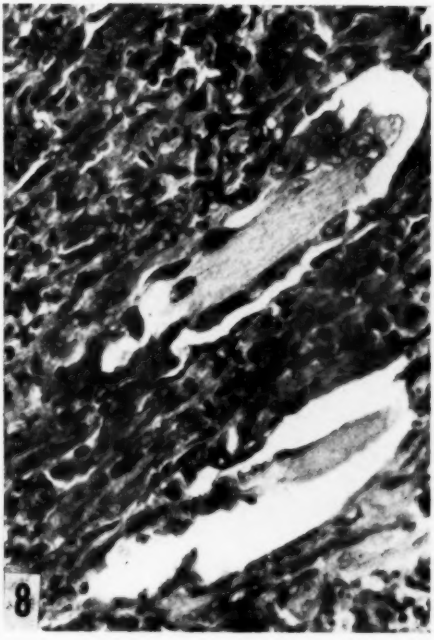
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Some Quantitative Observations on the Induction and Growth of Primary Pulmonary Tumors in Strain A Mice Receiving Urethan[1]

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Quantitative biologic investigations using the pulmonary tumor reaction in mice have been almost wholly limited to the enumeration of nodules observable on the surface of the lung (*1–3*). On the other hand, more detailed histologic studies (*4, 5*) have been concerned with histogenesis of the tumors and have not been quantitatively oriented. The studies of Rogers (*6*) and of Telford and Jeffery (*7*) indicate the usefulness of considering the size of the nodules and of the volume of the lung occupied by tumors. Additional information of this type appeared desirable for the understanding of the carcinogenic reaction.

The present investigation was designed with the following questions in mind: 1) What quantitative changes in regard to total volume and the total cell population occur in the lungs of strain A mice following a carcinogenic stimulus, and 2) What quantitative expressions describe the growth rate of the evoked pulmonary tumors? For these purposes, there is great advantage in using a carcinogenic stimulus that is effective following a single application. Urethan appears to be an ideal carcinogen in regard to the induction of pulmonary tumors, not only because one injection is sufficient but because it is eliminated from the animal within a day (*8*). The subsequent changes can thus be related to a single short event and not confused and complicated by cumulative, overlapping reactions that are produced when a carcinogen is administered over a considerable fraction or the whole period of the experiment.

Materials and Methods

Female mice of the inbred strain A were used. They were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine, in 2 shipments 2 weeks apart in the fall of 1953. Throughout the experiment they were maintained in plastic boxes with wooden shavings for bedding, and on a diet of Purina dog chow pellets with an unlimited supply of water.

Ethyl carbamate (urethan) was dissolved in water to make a 10-percent

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solution. A total of 110 mice were injected intraperitoneally with a single dose of 1.0 mg. per gram of body weight. At the time of injection of urethan, the mice were 6 to 10 weeks old and weighed 10.5 to 22.5 grams for a mean of 16.5 ± 2 grams.

Five to 15 mice were sacrificed by cervical dislocation at periods ranging from 1 to 133 days following urethan. Twenty mice, half of which were killed at the initiation of the experiment and the other half at 133 days, served as untreated controls.

The lungs were fixed immediately in a 10-percent formalin solution. After one day of fixation, the wet weights of both the total lung, from which the mediastinal tissues had been removed, and of the right major lobe were obtained on a torsion balance. The nodules visible on the surface of the whole lung were counted under strong illumination without magnification.

The right major lobe of the lung in five mice in each time period was sectioned serially, the sections being 5 micra in thickness. The sections were contiguous but did not represent the total tissue since the ends of the blocks were discarded. The sections were stained with hematoxylin and eosin for observation under the microscope.

Measurements of areas and of cell numbers were made with the aid of a Whipple micrometer introduced into the eyepiece. The Whipple micrometer is a grid containing 100 squares, each 1 mm. to the side. The whole grid is divided into four equal quadrants. As used by us, the micrometer was magnified tenfold by the eyepiece. Thus, at a total magnification of 970, each square covered a part of the slide whose area was $(1 \text{ mm.}/97)^2$, or 106.3 square micra.

Since the diameter of a cell is of the order of 10 micra, this unit and units derived from it were adopted for expressing the magnitudes of various quantities. Thus, 1 cu (cellular unit)$=10\mu$; 1 $\text{cu}^2=100\mu^2$; 1 $\text{cu}^3=1{,}000\mu^3$. With these units, the numerical value of a quantity shows the order of magnitude of the number of cells involved.

Throughout this paper it has been our intent to pay more attention to relative differences and to relative changes with time than to the absolute values of the measured quantities. This attitude tends to minimize the effects of systematic errors which may complicate the problem without being relevant to the questions under consideration. The following complications may be mentioned as illustrations: shrinkage caused by fixation and dehydration of tissues in the preparation of sections; uncertainty as to the state of expansion or contraction of lungs at the instant of fixation; error introduced when the volume of a tumor is calculated from the observed "equatorial area" by assuming a perfectly spherical structure.

General Cellularity

The lungs of mice killed 1 and 3 days following urethan appeared normal upon inspection. There was no evidence of cellular destruction, inflammatory reaction, or alteration in the morphology of individual cells.

The first morphologic change in the lungs of the mice was noticeable in sections obtained 7 days after urethan and definite in sections obtained 14 days after urethan. Statistical analysis, however, showed that a change began within the first day. The change was one of generalized disorientation and apparent increase in the number of cells in the alveolar walls (figs. 1 and 2). The alveolar walls were thickened, with the individual cells being oriented less regularly than in the alveolar walls of the normal lung. The appearance was one of diffuse hyperplasia and swelling. The stromal cells and the cells lining or supporting the capillary channels, as well as the cells lining the alveoli themselves, were involved in this generalized reaction. No clear differences from normal were noted in the appearance of individual cells.

Several quantitative measures of the change were used, two of which will be described.

The Whipple micrometer grid was placed at random along the subpleural areas of the sections of the lungs. The grid was oriented with one of the edges parallel to the pleura and one or two cellular layers under the pleura. Areas of obvious focal hyperplasia and of tumors were avoided in the study of the general hyperplasia.

The nucleated cells in each square of the grid were counted and recorded. Cells whose nuclei hit the upper and the right sides of the square were included, and cells whose nuclei hit the lower and left sides were excluded in the enumeration. Four to ten fields in sections of lungs from 3 to 5 mice were counted.

Column 2 of table 1 presents the results of the total counts. It is seen that an increase in the mean number of cells appears to begin within a day after urethan. This cellular increase reaches a peak at or near 28 days, when there is a 100-percent increase in the number of nucleated cells

TABLE 1.—*Indices of cellularity and mean weight of right lobe of lung as function of time after urethan*

(1)	(2)	(3)	(4)
Time after urethan (*days*)	Mean count per square*	Mean-three-highest counts per square*	Mean weight right lobe of lung (*mg.*)
0	0. 73	2. 7	86. 1
1	0. 85	2. 7	71. 8
3	0. 92	2. 7	83. 2
7	1. 11	3. 1	80. 2
14	1. 02	3. 1	78. 2
21	1. 35	3. 3	79. 9
28	1. 57	3. 8	86. 2
49	1. 33	3. 1	89. 0
84	1. 20	3. 1	90. 0
105	—	2. 9	98. 6
133	—	2. 8	92. 2
133†	—	2. 7	102. 8

*Magnification: 10 × 97 = 970. One square = 106.3 square micra.
†Controls; no urethan.

per unit area of the lung. The number of cells then gradually declines but is still significantly elevated at 84 days following urethan.

The visual impressions of cellular disorientation and of an increase in the number of small clusters of cells are not adequately reflected by the mean count of cells per square. Furthermore, this measure of hypercellularity may depend to some extent on the degree of expansion of the lung at the time it was fixed. For these reasons another index of cellularity was also examined.

The recorded fields of counts used in the evaluation of the first index of cellularity were re-examined and further determinations were added. In each of the four quadrants of the field the three largest counts per square were noted. The mean of the $12n$ counts obtained from n fields was taken as the second index of cellularity. This index, dependent as it is on the number of cell "mobs," is also less sensitive to the degree of expansion of the lung and thus approaches topological constancy.

The results are shown in the third column of table 1. The data simulate those shown in column 2 in that the index reaches its maximum at 28 days after urethan. As was to be expected, the "mean-three-highest" count is greater than the mean count.

Whether the cellular increase is due entirely to mitotic replication of the fixed-cell population of the lung, or represents, at least in part, an invasion by macrophages and other migratory cells is indeterminate from the present data.

Table 1, column 4, presents the mean weight of the right lobe of samples of five lungs and shows that the doubling in the number of cells was not reflected in an increase in the total weight of the tissue. This is not surprising since only the wet weight of the tissue after fixation was determined. Thus, the additional cells could replace an equal volume of alveolar water of practically the same density.

Hyperplastic Foci

In sections of the lung obtained 21 days after urethan, in addition to the general increase in cellularity and disorientation of cell relationships, there were relatively larger and more definite areas of cellular accumulation. These areas were encountered most frequently in subpleural and perivascular sites. They consisted mainly of cells that were not distinguishable from the cells seen in the alveolar walls of normal lungs but also contained some cells that were larger than normal (fig. 3).

These hypercellular areas are designated as "hyperplastic foci." It is obvious that no sharp distinction could be made between very small hyperplastic foci and aggregates of cells occurring in generalized hyperplasia. Distinction between hyperplastic foci and pulmonary tumors was possible on the basis of the adenomatous pattern of the tumors and of the larger cells comprising the tumors (figs. 3 and 4). Nevertheless, it can be taken for granted that some structures classified as hyperplastic foci may have been, in fact, diffuse tumors that were not recognizable as such.

In this connection it is of interest to compare cell counts in normal tissue, in urethan-treated tissue, in hyperplastic foci, and in tumors. In a number of hyperplastic foci and tumors the total number of cells seen in the equatorial section was counted. The mean density in cells per unit area was corrected to give the count per square of the Whipple micrometer at × 970. The following mean values were obtained.

The mean cell count in hyperplastic foci was 3.60 per square; in tumors, 2.35. The difference between these two counts is due chiefly to the fact that tumor cells are larger. The count in hyperplastic foci (3.6 cells per square) is approximately the same as the "mean-three-highest" count (table 1) in the general hypercellular tissue when the latter count is at its maximum at 28 days (3.8 counts per square).

Individual cells in hyperplastic foci and in tumors were measured, an attempt being made to find the equatorial diameters. Cells from normal lungs and from hyperplastic foci had a mean diameter of 0.67 cu, and from tumors, 0.88 cu. These values are not considered to have absolute accuracy but were obtained for comparative purposes. The mean diameter of tumor cells was larger than the mean diameter of the normal cells by a factor of 1.34. It follows that the mean volume was larger by a factor of 2.4. The mean nuclear volume was larger in the tumor cells than in normal cells by a factor of 3.4, reflecting the larger nucleus-cytoplasm volume ratio in tumor cells.

The mean number of cells in the hyperplastic foci and in the tumors at various times was estimated from the mean volumes of these structures, expressed in cu^3, and the mean cell-population density in the structures, expressed in cells per cu^3. The mean cell density was evaluated as follows: In a number of tumor sections and of hyperplastic foci sections, the total number of nuclei was counted, and in addition the area of the section was measured. The cells so counted occupied a cylinder whose base was the measured area and whose height was equal to the thickness of the section, augmented by twice the radius of the nucleus of a cell from a tumor or a hyperplastic area, respectively. From the volume and the number of cells, the cell density was calculated. The mean values obtained were 2.0 cells per cu^3 for tumors and 4.2 cells per cu^3 for hyperplastic foci. The corresponding volumes were 0.5 and 0.24 cu^3, respectively, per cell.

Size of hyperplastic foci.—Sections of the lungs were examined under low-power magnification and focal accumulations of cells were marked with ink. In general, the smallest recognizable contiguous *areas* contained approximately 35 cells. This corresponds to approximately 200 cells in the volume of the structure.

Having found a hyperplastic focus, consecutive sections in both directions were examined until the largest area of cross section was located. This "equatorial" area was drawn on a grid which was a magnified reproduction of the grid in the microscope. The area thus drawn was measured by means of a planimeter and the proper allowance was made for magnification.

In contrast to the tumors, whose shape was mainly spheroidal, the hyperplastic foci were mainly irregular structures. For our purposes, the structures observed were treated as spheres. From the area of the equatorial section, which was directly determined, the "radius" and the equivalent volume were calculated with the use of appropriate formulas.

The assumption of the largest area of cross section of a structure as the "equatorial area" of an equivalent sphere will increase the variance of volumes in a given sample of structures but need not necesssarily introduce serious bias of the mean value of the volumes in the sample. In fact, an analysis showed that any errors introduced by this procedure are apt to be relatively small in view of the very large variation of sizes within samples and the even larger relative changes occurring with progress of time. Nevertheless, since the mean area is an adequate measure of growth, and the areas were the directly measured quantities, the progress of growth is reported in terms of areas rather than in terms of the derived volumes.

Table 2 presents a summary of the observations on the size of hyperplastic foci as a function of time after urethan. The sample size, shown in the second column, offers a crude measure of the ease of finding hyperplastic focus areas on the slide sections. The range of sizes is shown in the third column. An area of 10 to 12 square cellular units is close to the lower limit of recognition for hyperplastic foci. From column 3 it appears as if new foci were being formed up to and possibly somewhat beyond the 49th day. On the other hand, it appears that no new foci were being formed by the 84th day, and that there is no further increase in size after that time. The geometric mean of sample size is shown in the fourth column. The geometric mean was adopted in preference to the arithmetic mean, both because the relative variation was large and also because inspection of the individual results suggested that a log-normal distribution would fit the results more adequately than a normal distribution.

The arithmetic mean volumes are shown in the fifth column. These values are used later for the estimate of the fraction of lung volume occupied by the hyperplastic foci.

TABLE 2.—*Size of hyperplastic foci in lungs as function of time after urethan*

(1)	(2)	(3)	(4)	(5)
Time after urethan (*days*)	Foci measured (*number*)	Area of foci, range (cu^2)*	Geometric mean of area (cu^2)	Arithmetic mean of volume (cu^3)
21	10	11–46	19.3	84
28	12	14–53	35.1	188
38	14	12–103	40.0	252
49	18	10–173	43.4	388
84	14	20–319	75.1	876
105	9	28–181	80.0	718
133	9	29–212	68.0	703

*cu=cellular unit, or 10 micra.

Number of hyperplastic foci.—The total number of hyperplastic foci as a function of time following urethan was calculated from sample analysis of random sections of the lungs.

Six sections of the right lobe were examined for each time interval. A record was made of the number of hyperplastic foci and of tumor areas seen in each section. From the mean value of this quantity and the previously obtained data regarding the mean size of the hyperplastic foci and of the tumors, it was possible to estimate the total number of hyperplastic foci and of tumors in the whole lung, as follows:

Let N be the mean number of spherical structures scattered at random throughout the lung. Let n be the mean number of cross-section areas seen in the slide sections. Clearly, n will depend on the following factors besides N:

S, the thickness of the section;
h, the minimum distance from the surface of the sphere if the area is to be recognized as hyperplastic focus or tumor;
A, the mean area of the section used for examination;
V, the mean volume of the whole lung;
R, the mean radius of the spheres.

Since n is the mean number of structures seen in a space whose area of cross section is A and whose thickness is $2R-2h+S$, it follows by proportion that the total number N in a lung whose volume is V is given by equation [1].

$$= \frac{nV}{A(2R-2h+S)} \qquad [1]$$

The quantities in equation [1] must be expressed in consistent units. The following values of the various quantities apply in our work: $S=5$ micra, or 0.5 cellular unit; we estimated the mean area of examined right lobe sections as 30 sq. mm., or 300,000 sq. cellular units; the mean volume of the lung, $V=250$ cu. mm.$=250 \times 10^6$ cubic cellular units; we estimated h to be approximately 1 cellular unit (the calculated value of N is rather insensitive to the assumed value of h); finally, the value of $2R$ was calculated from the mean values of the areas of the cross sections of the spheres at the respective times.

The results are presented in table 3. For the purpose of comparing the development of hyperplastic foci with that of tumors, corresponding data for tumors are also included in this table.

The numbers of hyperplastic foci and of tumors per whole lung are shown in the third and fifth columns, respectively. The following striking qualitative and quantitative differences between the two kinds of structure become apparent on inspection of the table.

The mean diameter of the hyperplastic foci approaches a fairly stationary value, whereas the tumors increase continuously. The number of hyperplastic foci reaches a maximum, then decreases. The number of tumors reaches an approximately constant value. Finally, the maxi-

TABLE 3.—*Number of hyperplastic foci and of pulmonary tumors per lung as function of time after urethan*

(1)	(2)	(3)	(4)	(5)
	Hyperplastic foci		Tumors	
Time after urethan (*days*)	Mean diameter (*cu*)*	Estimated mean number of foci per whole lung	Mean diameter (*cu*)	Estimated mean number of tumors per whole lung
14	3. 4	294	—	—
21	5. 2	450	—	—
28	6. 8	390	10. 6	15. 5
38	7. 4	610	—	—
49	8. 0	450	23. 6	37. 3
84	10. 4	260	38. 4	34. 8
105	10. 6	200	41. 8	35. 2
133	9. 8	83	52. 4	35. 7

*cu = cellular unit, or 10 micra.

mum number of hyperplastic foci is approximately 16 times as large as the eventual number of tumors.

Pulmonary Tumors

The earliest pulmonary tumors were detected in sections taken at 21 days following urethan. The recognition of pulmonary tumors was much more definite than that of hyperplastic foci, since the tumors were better circumscribed and were composed of larger cells that could be distinguished from the usual cellular components of the alveolar walls (figs. 5–10).

Unmistakable pulmonary tumors were difficult to find until sections were examined at 49 days after urethan. From then on there were progressively more tumor areas per slide, and the tumor areas were of progressively larger size. Except for rare tumors that had cuffs of lymphocytes at the periphery, the masses were devoid of inflammatory cells. The histologic appearance of the tumors was the same throughout the period of the experiment (figs. 5–10). None of the tumors showed central necrosis, cyst formation, or hemorrhage.

Size of tumors.—The size of tumors at various times was determined by the same procedure as was used in the measurements of hyperplastic foci. Data on 109 tumors are given in table 4 and in text-figure 1.

The number of tumors measured is shown in the second column of table 4. The numbers approximate the number actually present in the available slides.

The third column shows the ranges of values at various times. Allowing for random sample variation, the lower limit of the range appears to begin to increase after the 49th day, whereas the upper limit increases continuously. This suggests that new tumors continue to reach the minimum recognizable size up to the 49th day, but not beyond that; on the other hand, the pattern of the upper limit of the range indicates

TABLE 4.—*Size of pulmonary tumors as function of time after urethan*

(1)	(2)	(3)	(4)	(5)	(6)
Time after urethan (*days*)	Tumors measured (*number*)	Range of area of tumors (cu^2)*	Geometric mean of area, all tumors (cu^2)	Geometric mean of area of tumors 2, 3, and 4 (cu^2)	Arithmetic mean of volume (cu^3)
21	2	40–45	42. 5	43	209
28	4	78–95	87. 9	86	622
38	7	32–320	174. 0	273	2, 484
49	11	29–950	314. 4	783	9, 980
84	29	400–5, 200	1, 055. 0	2, 400	43, 900
105	28	160–3, 400	1, 247. 0	2, 410	46, 500
133	28	920–8, 500	2, 090. 0	3, 580	92, 300

*cu=cellular unit, or 10 micra.

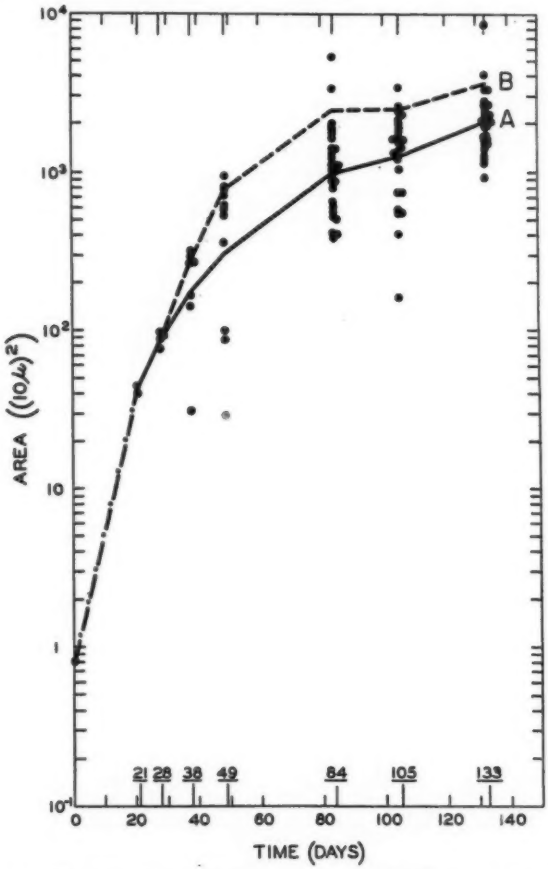
that once a tumor reaches a recognizable size it grows continuously. This impression is fortified by an examination of the last column of table 3, the data of which indicate that the eventual and final number of tumors is reached on or near the 49th day. In this connection, it is worth mentioning again that the number of hyperplastic foci reaches a maximum in the vicinity of 38 days (table 3); that the lower limit of the size range of hyperplastic foci begins to increase beyond the minimum recognizable size after the 49th day (table 2) and that the upper limit of range stops increasing at about the same time.

The fourth column of table 4 shows the progress of the geometric mean of the observed tumor areas as a function of time. These data are shown as curve A of text-figure 1. This curve does not necessarily simulate the growth curve of a single tumor for the following reasons.

For the sake of illustration, let it be assumed that each tumor passes a critical stage at which time its characteristics undergo a sudden and sharp change; let this change be reflected in a sharp change in the growth rate, imparting a cusp to the growth curve. If rates and times of critical change are randomly distributed in a sample, the progress of the sample mean size will not only fail to yield any quantitive information on the nature of the critical stage; worse yet, the sample mean will fail to show even qualitatively the existence of a critical stage. In general, the sample mean growth curve is not apt to disclose the finer points of the individual growth curve.

There is yet another factor which limits the usefulness of curve A for a quantitative interpretation of the growth of tumors. The sample sizes, shown in the second column of table 4, reflect the fact that only a few of the eventual tumors are recognizable as such at the earlier times. It follows that the means of the *observed* tumors do not represent the means of the same population, except possibly toward the end of the experiment, when all the tumors are large enough to be recognizable. Thus, curve A is not a population-growth curve in the usual sense of the term.

At the early stages of the experiment, when a large majority of the eventual tumors are still unrecognizable, there are only a few tumors large enough to be observed. Assuming, then, that attention is centered at all times on, say, the mean of the largest four of the observed tumors, the progress of this mean will represent a true growth curve of the faster-growing part of the population of tumors. In order to decrease the extent of sample variation, we studied the mean of the tumors whose ranks at each period were Nos. 2, 3, and 4. The mean "second-third-and-fourth-areas" is shown as a function of time both in the fifth column of table 4 and as curve B of text-figure 1. Since only two tumors were measured at 21 days, their mean is listed and is assumed to be somewhat larger than the desired mean.

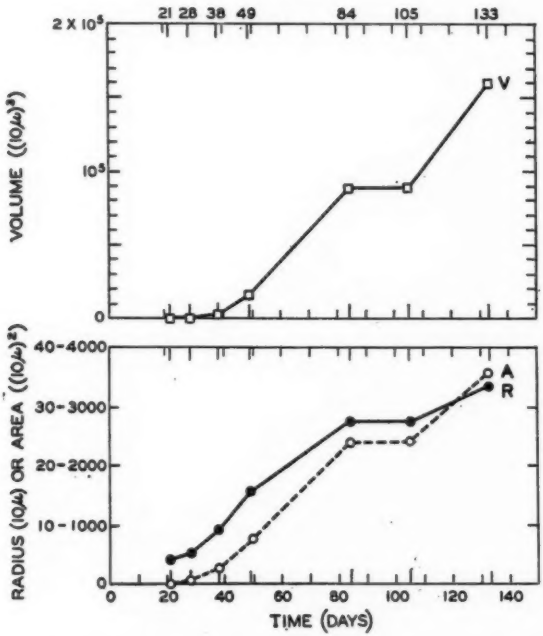


TEXT-FIGURE 1.—The growth of pulmonary tumors evoked by a single intraperitoneal injection of urethan, 1 mg. per gram body weight, in strain A mice. Curve A shows the geometric means of the tumors. Curve B shows the means of largest tumors Nos. 2, 3, and 4. The point at zero time represents the equatorial area of a sphere whose volume equals the mean volume per cell in a tumor.

Curve B of figure 1 shows approximately exponential behavior up to 49 days, with a mean volume-doubling time of 4.4 days between 28 and 49 days. Eventually, however, the relative rate decreases drastically. The mean doubling time between 49 and 84 days is 14.5 days, and between 84 and 133 days it is 55.5 days.

Attempts to describe the entire growth process by some single function were not successful. In particular, it should be mentioned that the theoretical course discussed by Mayneord (*9*) will not fit curve B. In Mayneord's model, as the sphere becomes large, growth begins to be limited to an outer layer of the sphere, and the rate of change of the radius approaches a constant value.

The slope of the curve on the semilog field of text-figure 1 is a measure of the instantaneous *relative* rate of growth. It is clear from the diagram that the relative rate decreases with time, but the diagram does not portray clearly the progress of the absolute rate of change in size. Subject to random sample errors, the experimental data indicate that beyond 49 days and up to 133 days following urethan the absolute rate of increase of the geometric mean volume is fairly constant. As a necessary mathematical consequence, the absolute rate of growth of the equatorial area decreases slowly, and the absolute rate of growth of the radius decreases somewhat faster (text-fig. 2).



TEXT-FIGURE 2.—Temporal course of geometric mean radius, area and volume of the second, third, and fourth largest pulmonary tumors. *V* is volume, *A* is area, and *R* is radius.

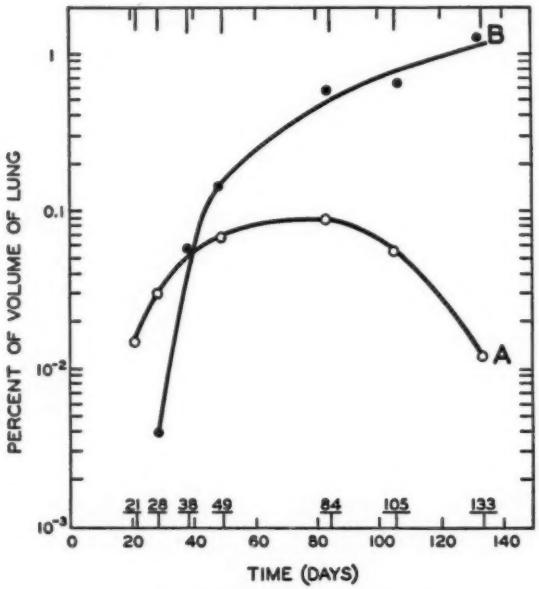
Number of tumors.—The mean total number of tumors per lung as a function of time was calculated by the same procedure as was used for similar determinations of hyperplastic foci.

Column 5 of table 3 presents the calculated number of tumors per lung. It appears that the number of recognizable tumors increases rapidly at first, but that the full quota of tumors seems to be reached at or near the 49th day after urethan. The number remains approximately the same from the 49th to the 133rd day, whereas the size of the tumors increases progressively.

From the values of the arithmetic mean of the volume of the tumor as a function of time (table 4), and from the calculated total number of tumors as a function of time (table 3), it is possible to determine the fraction of total lung volume occupied by the tumors and by the hyperplastic foci, as a function of time. The data are plotted in text-figure 3. In these calculations, the mean volume of the lung was taken as 250 mm.[3]

The volume occupied by tumors increases steadily; however, at all times this constitutes only a small fraction of the total lung volume. At 49 days tumors occupy approximately 0.1 percent of the total volume. Even at 133 days only 1 percent of the total volume is occupied by tumors.

Even though the number of hyperplastic foci is much larger, their size is such that they occupy a negligibly small total volume at all times. At its maximum, the total volume occupied by the hyperplastic foci constitute only 0.1 percent of the lung volume.



TEXT-FIGURE 3.—Fraction of total lung volume occupied by pulmonary tumors and by hyperplastic foci as a function of time after urethan. Curve A represents volume occupied by hyperplastic foci; curve B, by pulmonary tumors.

It is not surprising, therefore, that the presence of tumors and of hyperplastic areas at various stages of the experiment was not reflected in an increase in the weight of the right lobe of the lung (*cf.* table 1).

These data can be expressed in terms of cells as follows: Appropriate calculations indicate that there are approximately 200 million cells in a mouse lung. Thirty-six tumors per lung were evoked. If each tumor arose from a single cell, the successful carcinogenic transformation involved one cell in a population of 5 to 6 million. The number of cells in a mean pulmonary tumor at 133 days following urethan is approximately 144,000 (text-fig. 4). The total number of cells in the 36 lung tumors is approximately 5 million. Thus at 133 days tumor cells comprise approximately 2.5 percent of the cell population of the lung.

Surface tumors.—The number of pulmonary nodules observed on the surface of the lungs as a function of time is recorded in table 5.

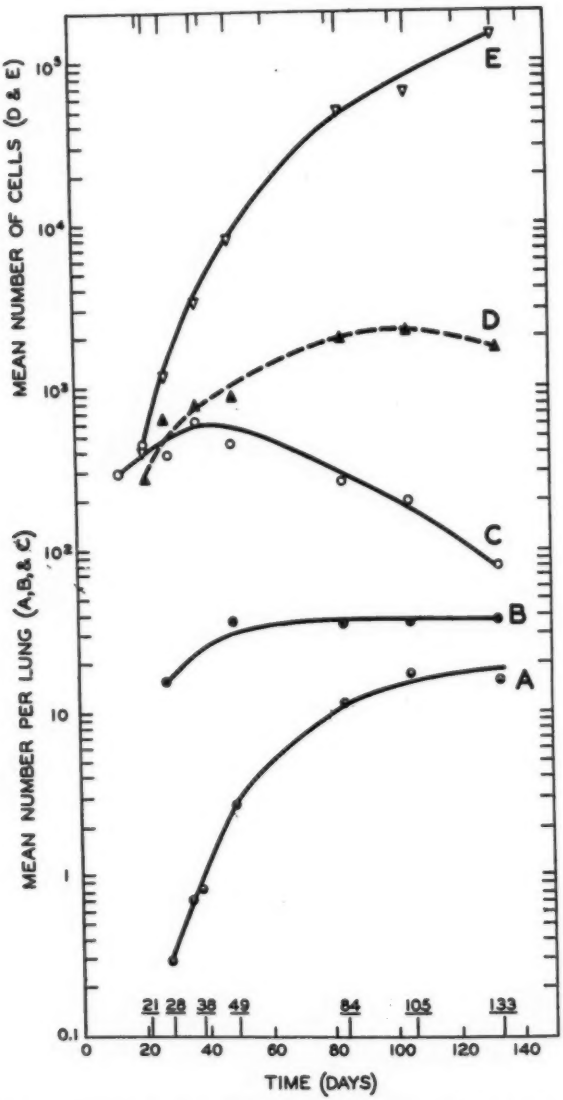
Analysis of the data summarized in table 5 showed that, allowing for the small sample size employed in the present series, the results conform well with the compound Poisson distribution observed previously in other series (*2*). At low values of the mean number of tumors per animal, the variance equals approximately the mean number of tumors. As the mean number of tumors increases, the variance also rises, but at a faster rate.

Curve A of text-figure 4 shows that the number of tumors visible on the surface continues to increase for at least up to 105 days, whereas observations of the sections under the microscope indicate that the total number of tumors becomes approximately constant by 49 days. The total number of pulmonary tumors is calculated to be approximately 36, whereas only 17 and 15, respectively, were counted on the surface at 105 and 133 days. Thus, approximately half the total tumors are visible on the surface to the naked eye by 105 days.

TABLE 5.—*Pulmonary tumors visible on the surface as function of time after urethan*

(1)	(2)	(3)	(4)	(5)
Time after urethan (*days*)	Number of mice	Mean number of tumors	Variance	Distribution
0	10	0	0	0(10)
1–25	45	0	0	0(45)
28	15	0. 27	0. 35	0(12), 1(2), 2
35	13*	0. 69	0. 57	0(6), 1(5), 2(2)
38	5	0. 80	0. 70	0(2), 1(2), 2
49	10	2. 7	4. 2	0, 1(2), 2(2), 3(3), 5, 7
84	10	11. 2	16. 1	5, 7, 8, 9, 11, 12, 13, 14, 15, 18
105	5	17. 0	39. 0	9, 12, 20(2), 24
133	5	15. 4	25. 5	8, 15(2), 17, 22
133†	10	0. 1	0. 1	0(9), 1

*15 originally; 2 mice discarded due to presence of pneumonitis.
†Controls; no urethan.



TEXT-FIGURE 4.—Composite figure of the relationships of numbers of pulmonary tumors and hyperplastic foci, size of tumors and hyperplastic foci, and number of cells in tumors and hyperplastic foci as functions of time after urethan. Curve A represents the mean number of surface tumors per lung; curve B, the mean number of tumors per lung observed microscopically; curve C, the mean number of hyperplastic foci per lung; curve D, the number of cells per mean hyperplastic focus; curve E, the number of cells per mean pulmonary tumor.

The continuous increase of the number of tumors observed on the surface long after the administration of urethan is not due to the genesis of new tumors. Many of the tumors are too small to be recognized, or are situated too deep from the surface, and are recorded as new tumors when they have grown to larger size or to closer proximity with the surface.

The enumeration of tumors by surface count without magnification is so much simpler and faster than the examination of microscopic sections that wherever possible it is desirable to use this method, provided the limitations of this method are kept in mind. The chief limitation stems from the fact that the surface count without magnification does not contain a representative sample of the entire tumor population, since this count recognizes only those tumors which have passed beyond some minimum size. The effect of this selective sampling is best indicated by an illustration.

Consider an experiment in which two animal samples are subjected to two different experimental procedures, A and B. Merely for illustrative purposes, let it be assumed that both procedures evoke the same mean number of tumors per animal, but that the tumors grow faster under procedure B. In these circumstances, the surface count will be larger in sample B, and the erroneous conclusion may be drawn that procedure B evokes a larger number of tumors. On the other hand, if the two procedures evoke significantly different numbers of tumors but do not affect the rates of growth of the tumors, the differences between the numbers of evoked tumors will be correctly reflected in the differences between the numbers of observed surface tumors.

Discussion

We have presented some quantitative observations of changes that occur in the lungs of strain A mice following a single intraperitoneal injection of 1 mg. of urethan per gram body weight.

Statistical evidence indicates that within the first day, and reaching a peak at approximately 4 weeks, there was a progressive increase in the total number of nucleated cells in the lung. The total cell number was doubled at 4 weeks. Beyond this time the generalized cellularity subsided slowly, approaching normal levels by 15 weeks after urethan.

In addition to the generalized hypercellularity, there appeared by 3 weeks circumscribable regions of accumulations of cells. Most of the individual cells within this region could not be distinguished from the normal cell population of the lung; however, a few cells appeared to be larger than the normal cells. These hyperplastic foci increased in number and reached a peak of approximately 600 per animal at approximately 5 weeks. The number of hyperplastic foci then decreased gradually. The mean size of the hyperplastic foci increased for approximately 12 weeks and then decreased very slowly.

The first histologically recognizable pulmonary tumors, made up of large cells in adenomatous arrangement, were seen in sections taken 3 weeks after urethan. The number of tumors increased rapidly up to 7

weeks, and from then on remained approximately the same. The total number of tumors evoked by the 1 mg. per gram dose of urethan was approximately 36 per animal.

The mean size of the pulmonary tumors increased continually. Growth proceeded through a rapid phase for approximately 7 weeks. From 7 to 19 weeks the relative growth rate became much slower, although the absolute rate of growth of the volume remained fairly constant throughout this period.

At the termination of the experiment, 19 weeks after urethan, only 1 percent of the lung volume was occupied by pulmonary tumors. This represented only 2.5 percent of the total number of cells in the lung.

Additional data regarding the growth of urethan-induced pulmonary tumors were available from two other investigations. In the first,[4] strain A mice were killed 30 weeks following a single intraperitoneal dose of urethan, 1 mg. per gram body weight. Thirty-five pulmonary tumors were measured. It was found that the geometric mean of the area was 3,300 cu^2; the geometric mean of tumors 2, 3, and 4 was 5,800 cu^2, and the geometric mean of the volume of the latter was 336,000 cu^3. The pulmonary tumors at 30 weeks occupied 4.5 percent of the total lung volume. These findings fit well the observations recorded in text-figures 1 and 2, and allow extension of the conclusions regarding the growth of pulmonary tumors to 210 days following urethan.

The second experiment provided 14 pulmonary tumors in strain A mice killed 12 and 15 weeks following a single intraperitoneal dose of urethan, 0.25 mg. per gram body weight. The mean numbers of pulmonary tumors at those time periods were approximately one fourth the number evoked by the 1.0 mg. per gram dose, whereas the mean sizes reached were approximately the same as the mean sizes following the larger dose. This would indicate that the dose of urethan controls the induction of the tumors but not the subsequent growth of the tumors. A similar conclusion on the basis of other experiments and considerations was reached by Rogers (*6*).

A comparison of the observed sizes and numbers of tumors and of hyperplastic foci is of considerable interest, since it may have an important bearing on the genesis of the tumors. The question at issue is whether the hyperplastic foci are precursors of tumors or whether the latter arise independently of the hyperplastic reaction.

The observations at 21 and 28 days contribute relevant information on this point. The numbers of recognized tumors at these two times are relatively small as compared with the numbers recognized at later times (*cf.* table 4, column 2). The obvious explanation that the remaining tumors were too small to be recognizable at 21 and 28 days is not acceptable for the following reasons. The ranges of observed tumor areas at 21 and 28 days were, respectively, 40 to 45 and 78 to 95 cu^2. In each case the lower value of the range was significantly larger than the minimum

[4] We are indebted to Dr. B. L. Friedlander for these animals, controls of one of his experiments.

recognizable area, which was approximately 10 cu^2. Table 4 shows that none of the observed tumors had an area smaller than 29 cu^2.

These observations are consistent with the assumption that during the earlier stages of its growth a tumor possesses the same visible characteristics as the hyperplastic foci. A hyperplastic focus seemingly becomes recognized as a tumor when the equatorial area of the hyperplastic focus is 30 cu^2 or larger.

Some support to this conclusion may be obtained from an examination of curves D and E of text-figure 4: At 21 days, the mean number of cells per tumor was larger than the mean number of cells per hyperplastic focus by a factor of a little less than two. This close relation between the two mean cell numbers may be accidental; on the other hand, it is consistent with the assumption that the tumors observed at 21 days represent structures arising from the hyperplastic foci.

The evidence that hyperplastic foci are precursors of tumors is admittedly circumstantial and requires further investigation. Should it be definitely proved that tumors obligatorily arise from areas of focal hyperplasia, then the following question demands clarification.

The maximum number of hyperplastic foci per animal was approximately 600, as compared with a total of 36 tumors per animal. Were 36 of the 600 foci essentially different from the others without exhibiting any obvious morphologic differences? Or were all 600 foci potential tumors or precursors that required some additional stimulus which shifted one sixteenth of the hyperplastic foci toward full development into tumors?

The question whether hyperplastic foci are obligatory precursors of pulmonary tumors or whether tumors arise by a parallel, independent path is of fundamental importance. It would seem that a concurrent study of tumors and of hyperplastic foci under widely varying experimental conditions should reveal the nature of the possible association. The necessary experimental variants include the detailed quantitative examinations of pulmonary tumors and hyperplastic foci in less susceptible strains of mice and the quantitative examination of both structures following a wide range of doses of urethan, including different dose schedules.

Should it be found that tumors are not related causally to hyperplastic foci, the situation would be similar to the relation between hepatomas induced by carbon tetrachloride and the accompanying cirrhosis and tissue regeneration. Eschenbrenner and Miller (*10*) have shown in their careful study that, whereas there is a correlation between tumor formation and cirrhosis, the latter is probably not obligatory for the induction of the hepatomas.

One of our main interests in the growth rate of pulmonary tumors was to provide material that might elucidate whether there is a true latent period in the induction of these neoplasms, and whether the tumors arise from single cells or from groups of cells. Were the growth rate of the pulmonary tumors exponential throughout the period of observation,

this question could have been answered by extrapolation of the logarithmic line back to zero time to see whether the ordinate at zero time approximated the area to be allotted to a single cell. The complex nature of the growth rate of these organoid neoplasms, however, makes such extrapolation hazardous. Inspection of text-figure 1 certainly suggests that the largest tumors grew without a period of latency. On the assumption that the law of growth of the largest tumors differs quantitatively but not qualitatively from the law of growth of the other tumors, it seems very likely that the other tumors begin to grow without an appreciable latent period.

It should be emphasized that the investigation covered a period during which the number of cells comprising the pulmonary tumors increased from a minimum of 200 to a maximum of 200,000. During the same period, the relative rate of growth decreased from a mean doubling time of 4.4 days between 4 and 7 weeks to a mean doubling time of 55 days between 12 and 19 weeks. The tumors at the termination of the observations showed no central necrosis, hemorrhage, or other morphologic explanation of the decrease in the relative rate of growth.

It may be useful to mention some possible mechanisms which may be involved in the gradual decrease in the relative growth rate of the tumors. It is conceivable, for example, that the rate of growth of an individual tumor is controlled by its own size: the larger the tumor becomes, the smaller the relative rate of its further increase. If this were the mechanism controlling the relative rate of growth, then the growth of the smaller tumors would be decelerated more slowly than that of the larger tumors, and there would be a general "closing up of ranks" with the progress of time. An inspection of text-figure 1, however, shows no obvious trend toward a lower range of values. The points for a given time tend to be confined to a range of approximately 1 logarithmic unit. The corresponding range for the volumes would be approximately 1.5 logarithmic units, which is equivalent to a factor of approximately 30. It may be concluded that the self-limiting effect of size is not shown to be the factor which controls the rate of growth of individual tumors.

A modification of the above mechanism would postulate the control of growth by the over-all total tumor volume within the lung of a given animal. Assuming that each tumor elaborates some inhibitor, or some substance that stimulates the production of an inhibitor by the host, the amount of which is proportional to the total tumor tissue volume, it would follow that the general growth rate would decrease with the progress of time. The relative rates of growth of small and large tumors would be equally affected, and the range of sizes would be approximately unchanged with time.

The following experimental evidence, however, appears to be contrary to this interpretation. One-fourth dose of urethan induced approximately one-fourth the number of tumors, which grew at the same rate as tumors evoked by the larger dose. If tumors elaborated some inhibitory substance, the mean size of the tumors following the smaller dose should have

been larger. The observations of Rogers (*6*) also show that the size distribution of tumors among animals with a few tumors is not conspicuously different from the size distribution among animals with very many tumors.

Another possibility that should be mentioned is that the growth of the individual tumor passes through two distinct stages—an early rapid stage and a late slow stage—because another consecutive change occurs in the cells comprising the tumors. The individual tumor growth curve would be represented by a broken curve consisting of an initial steep branch and a final slowly rising branch. Because of variations in speed and in the time at which the cells of individual structures reach the second alteration, the mean tumor growth curve would be a curve without a cusp, as indicated in text-figure 1. This explanation would imply that the single injection of urethan initiates not one sudden change, but sets in motion a series of at least two cellular changes, the second one occurring some 7 weeks after the first.

Summary

Quantitative histologic observations were made of lungs of strain A mice killed from 1 to 133 days following a single intraperitoneal injection of 1 mg. of urethan per gram body weight.

Within a day, and reaching a peak at approximately 4 weeks, there is a generalized increase in the number of nucleated cells in the lung. By 3 weeks there appear circumscribable hyperplastic foci, and by 5 weeks there are approximately 600 such foci per animal. The number of hyperplastic foci then gradually decreases.

The first pulmonary tumors are recognized in sections taken 3 weeks after urethan. The number increases rapidly to 36 per lung at 7 weeks, and then remains at that level. The mean size of the tumors increases rapidly. The mean doubling time of the larger tumors is 4.4 days up to 7 weeks, after which the relative rate decreases to a mean doubling time of 55.5 days between 12 and 19 weeks. The absolute rate of change in the geometric mean of the volume, however, remains fairly constant between 7 and 19 weeks. At 19 weeks, pulmonary tumors occupy only 1 percent of the lung volume and represent 2.5 percent of the total cell population of the lung.

The possible implications of the findings were discussed.

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PLATE 6

Strain A mouse lung. Hematoxylin and eosin. × *760*

FIGURE 1.—Normal mouse lung, showing detail of alveolar wall.

FIGURE 2.—Lung at 28 days following urethan, showing generalized hypercellularity and disorientation.

FIGURE 3.—Area from hyperplastic focus, 38 days following urethan.

FIGURE 4.—Edge of pulmonary tumor, 105 days following urethan.

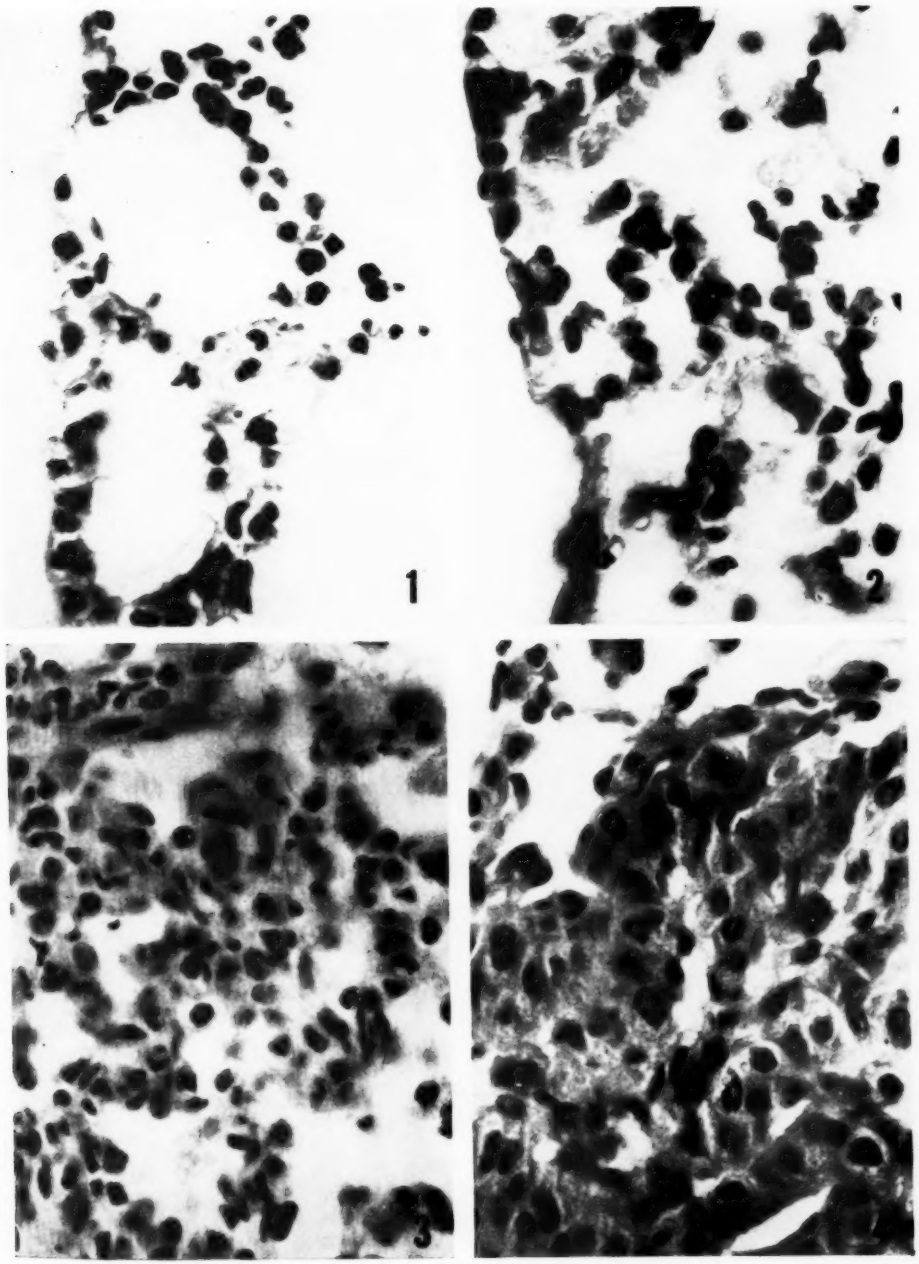

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PLATE 7

Pulmonary tumors induced with single intraperitoneal dose of 1 mg. of urethan per gram body weight. Hematoxylin and eosin.

FIGURE 5.—At 21 days. × 160

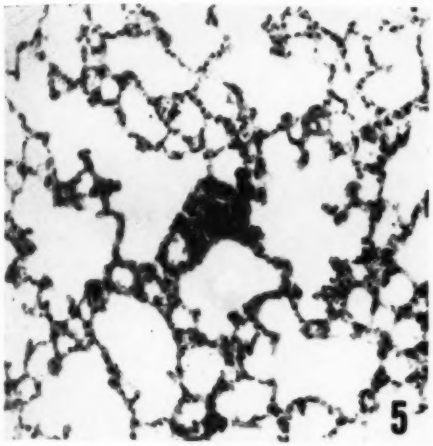
FIGURE 6.—At 38 days. × 80

FIGURE 7.—At 49 days. × 80

FIGURE 8.—At 84 days. × 80

FIGURE 9.—At 105 days. × 80

FIGURE 10.—At 133 days. × 80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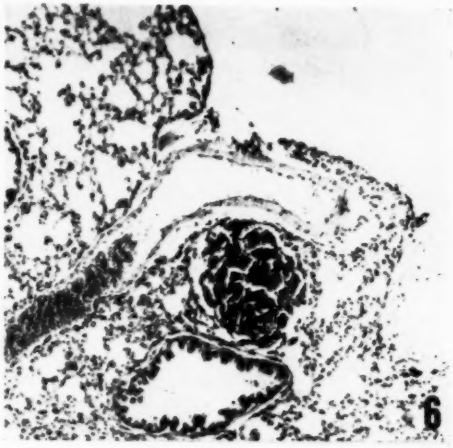
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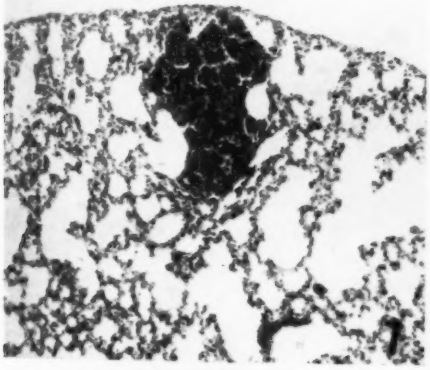
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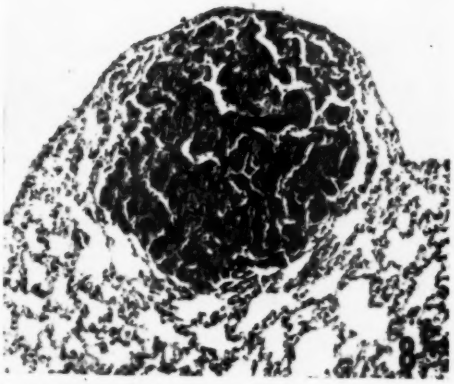
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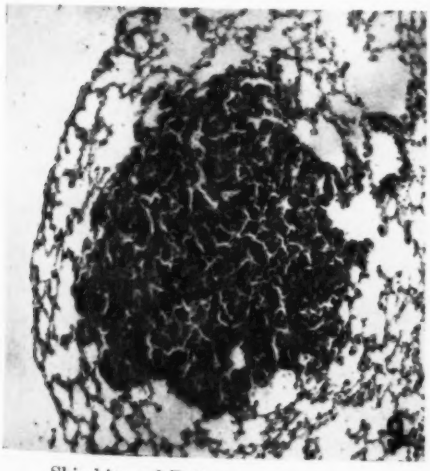
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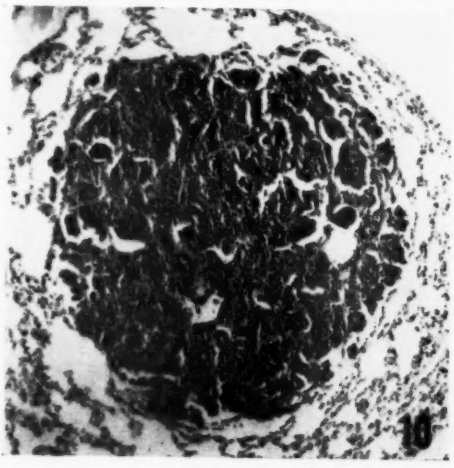
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Enzyme Changes Induced in Normal and Malignant Tissues with Chemical Agents. V. Effect of Acetylpodophyllotoxin-ω-Pyridinium Chloride on Uricase, Adenosine Deaminase, Nucleoside Phosphorylase, and Glutamic Dehydrogenase Activities [1]

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In previous publications (*1–3*) from this laboratory on enzyme changes induced with tumor-necrotizing agents in normal and malignant tissues of mice, we have shown a marked decrease in cytochrome oxidase activity, cytochrome c, succinoxidase activity, and diphosphopyridine nucleotide (DPN) in mouse tumors soon after the administration of these agents. Since all these components of the cytochrome system are associated with the mitochondrial fraction of the cell, and since disruption of mitochondria by physical means results in an appreciable loss in the activity of certain of their enzymatic components, such as cytochrome oxidase and succinoxidase (*4, 5*), the relationships of the induced biochemical changes following injection of tumor-damaging agents to some of the morphologic changes produced were investigated.

Little information is available with respect to the morphologic changes produced in mitochondria. This is due in part to the fact that stains, such as Janus green, which are used for the differential staining of these structures, are dependent on the oxidizing enzymes concentrated in the mitochondria. With the marked reduction in these enzymes in chemically damaged tumors, these vital stains apparently lose their staining specificity for mitochondria.

In order to further assess the significance of the loss of cytochrome oxidase and of related enzymes in relationship to any morphologic changes in the intracellular structure, a few of the enzymes associated with either the mitochondrial or supernatant fractions of the cell were studied. The enzymes investigated were uricase and glutamic dehydrogenase, mitochondrial enzymes, and the soluble, nonsedimentable enzymes found in the supernatant, *e.g.*, adenosine deaminase and nucleoside phosphorylase.

Methods and Materials

The tumor-damaging compound employed in these investigations was *acetylpodophyllotoxin-ω-pyridinium chloride* (NCI-3022), a soluble derivative (*6*) of podophyllotoxin.

[1] Received for publication December 3, 1954.
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[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

CAF_1 [(BALB/c × A)F_1] hybrid mice bearing 6-day-old intramuscular implants of Sarcoma 37, or strain C3H mice bearing 2-week-old implants of Hepatoma 129, were employed in these experiments. The tumor-bearing mice received a single subcutaneous injection of the compound (NCI-3022) and were sacrificed, in groups of at least 4 animals each, at intervals of 2 to 24 hours after injection. Homogenates of normal and tumor tissues were prepared as described previously (*7*) for enzyme studies.

Activity of purine-metabolizing enzymes, such as uricase, adenosine deaminase, and nucleoside phosphorylase in tissue homogenates, was determined spectrophotometrically according to the procedures used by Schneider and Hogeboom (*8*). In the case of uricase it was found necessary to use freshly prepared uric acid each time the determination was made.

The glutamic dehydrogenase activity of the homogenates was determined spectrophotometrically at 340 mμ by measuring either the rate of reduction of DPN in the presence of glutamate or by the rate of oxidation of $DPNH_2$ (dihydrodiphosphopyridine nucleotide) in the presence of α-ketoglutarate and ammonium ions. Hogeboom and Schneider have reported that the glutamic dehydrogenase activity of homogenates was greatly increased by procedures causing disruption of mitochondria (*9*). In the present experiments the homogenates prepared in 0.25 *M* sucrose solution were forced through a small orifice under 20,000 psi[4] at 3° C. in a stainless steel apparatus described by Milner *et al.* (*10*).

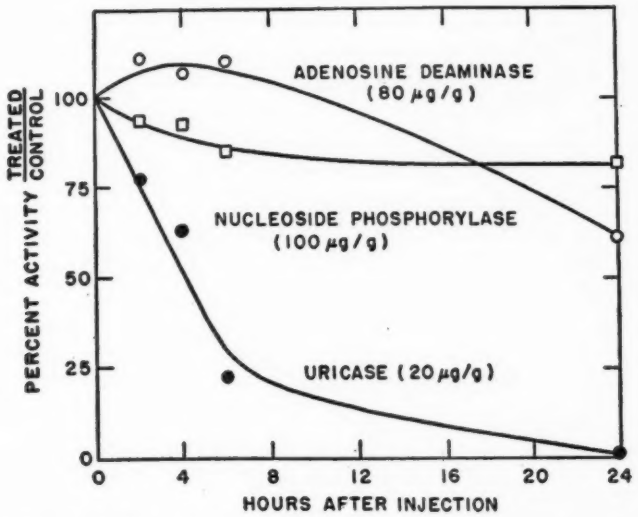
Results

A marked loss in uricase activity in homogenates of Sarcoma 37 (text-fig. 1) was observed soon after the subcutaneous injection of the compound (20 μg./gm.); about 75 percent of original activity was lost in 6 hours and almost all the activity disappeared in 24 hours. The pattern of reduction of uricase activity was similar to that of succinoxidase activity reported in a previous paper (*2*) in which the dose level of the compound was the same as in the present studies.

Only small changes were noted in the activity of adenosine deaminase and nucleoside phosphorylase of the tumors (text-fig. 1). Less than 20 percent of their activities was lost during the first 6 hours; and at 24 hours after injection a diminution of only 20 percent of the nucleoside phosphorylase activity and 40 percent of the adenosine deaminase activity was observed, even following a lethal dose (80–100 μg./gm.) 4 to 5 times that used for uricase determinations.

The enzyme activities (uricase, adenosine deaminase, and nucleoside phosphorylase) in homogenates of liver from the treated animals were unchanged. Similarly, incubation *in vitro* of Sarcoma 37 homogenates with as much as 250 μg. of NCI-3022 per ml. of medium failed to produce any significant changes in any of the enzyme activities.

[4] psi = Pounds per square inch.



TEXT-FIGURE 1.—Effect of NCI-3022 on three purine-metabolizing enzymes of Sarcoma 37.

Control values per 100 mg. fresh tissue per minute for—

Adenosine deaminase (micromoles of adenosine deaminated) 0.074–0.082 nucleoside phosphorylase (micromoles of uric acid formed from inosine) 0.11–0.13.

Uricase (micromoles uric acid destroyed) 0.013–0.014.

In the case of glutamic dehydrogenase, little information was available in the literature on the activity of tumor homogenates. Consequently, the activity of this enzyme in some of the tumors commonly used in this laboratory was determined. These tumors, together with their activities, are listed in table 1, which gives the activity for both directions of the reaction, *viz.*, glutamate$\rightleftharpoons$$\alpha$-ketoglutarate as described in "Materials and Methods." All the values are for homogenates forced through a tissue press at 20,000 psi. Values in tumors for the reaction α-ketoglutarate$\rightarrow$glutamate were about 2 to 3 times greater than for the reverse reaction. This is similar to that found for liver by other investigators (*9*) as well as by us. Values for glutamic dehydrogenase in tumors other than hepatoma were 1/12 to 1/30 that of liver. Hepatoma showed an activity about 3 to 7 times that of other tumors and about 1/3 to 1/4 the activity of normal liver.

The effect of NCI-3022 on glutamic dehydrogenase activity of Hepatoma 129 is shown in text-figure 2. The activity of the enzyme was determined on homogenates of hepatoma from animals injected with the drug, both before and after passing through a hydraulic press under 20,000 psi. The results show that the effect of the compound on the enzyme activity of the homogenates was the same whether it was passed through a press or not. Changes in the enzyme activity of the tumor were small for the first few hours after injection of the drug. However,

TABLE 1.—*Glutamic dehydrogenase activity* of some tumor-tissue homogenates*

Tumor	Enzyme activity†	
	I DPN⟶DPNH$_2$	II DPNH$_2$⟶DPN
Sarcoma 37	0.008	0.010
Sarcoma 180	.005	.017
Adenocarcinoma C_3HBA	.004	.024
Adenocarcinoma 241-6	.005	.031
Carcinoma 63	.007	.011
Lymphoma 1	.004	.012
Lymphoma 2	.007	.013
Lymphoma MOL_4‡	.005	.016
Leukemia 1210	.007	.020
Harding-Passey melanoma	.003	.024
Hepatoma 129	.021	.068
Liver	.090	.210

*Activity of homogenates passed through tissue press under 20,000 psi.

†Expressed as μM DPN reduced per minute per mg. nitrogen in column I, or μM $DPNH_2$ oxidized per minute per mg. nitrogen in column II.

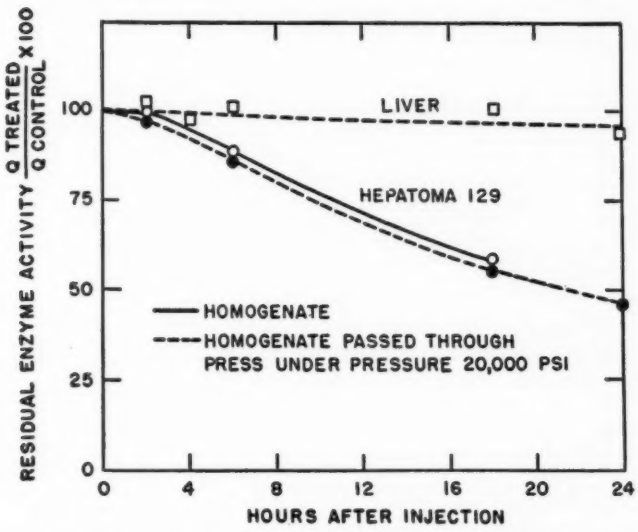
‡Originated in strain DBA mouse in this laboratory and transplanted for 40 generations.

18 hours after injection the loss in enzyme activity was about 40 percent. Under similar conditions the enzyme activity of the liver was not affected to any appreciable extent.

Discussion

Most of the biochemical changes described previously by us (*1–3*) following a single injection of tumor-damaging compounds have been reflected in a marked decrease of such components as cytochrome oxidase, cytochrome c, DPN, succinoxidase, etc. Some of these properties have generally been associated with particulate structures of the cell such as mitochondria. Since decreases in some of these components can be produced by physical means, such as disruption of particulates by sonic vibration or by passing homogenates through a press (*4, 5*), some of the relationships of the changes produced after treatment with tumor-damaging drugs to those produced by nonspecific damage to cellular components were investigated.

Enzymes, such as uricase and glutamic dehydrogenase, were selected for this purpose because the effects of mechanical disruption of cell particulates on these enzymes did not parallel those produced on cytochrome oxidase or succinoxidase. Uricase activity is concentrated in the mitochondria, but Schneider and Hogeboom (*8*) have demonstrated that



TEXT-FIGURE 2.—Effect of NCI–3022 (20 μg./gm.) on glutamic dehydrogenase activity of Hepatoma 129 and of liver.

Control values in terms of micromoles of $DPNH_2$ oxidized per minute per mg. *N* for tissue homogenates passed through press under 20,000 psi.

Hepatoma	0. 063–0. 068
Liver	0. 202–0. 210

virtually all of it can be recovered in the sedimentable fraction after disruption of mitochondria by physical means. Following a single injection of NCI-3022, however, virtually all (98%) of the uricase activity was lost.

In the case of glutamic dehydrogenase, as well, Hogeboom and Schneider (*9*) have shown that most of the activity of this enzyme is in the mitochondria. Breaking up of the mitochondria, in this instance, produces a large increase in glutamic dehydrogenase activity and solubilizes the enzyme. They have reported as much as a 700-percent increase (in sucrose solutions) in liver, and in our own experiments we have obtained about a 400-percent increase in liver and in tumors. Following injection of NCI-3022, however, only a *decrease* in this enzyme was observed. Twenty-four hours after injection of the drug the glutamic dehydrogenase activity of tumor-tissue homogenates was only 46 percent of that of the tissue from untreated animals. Forcing the tissue homogenates from treated animals through a press produced a fourfold increase in enzyme activity similar to that observed with untreated tissue, suggesting that a large proportion of the mitochondria was in a relatively intact form. Although most of the information on the locus of glutamic dehydrogenase is available only for liver, it may be presumed that the enzyme activity is located mainly in the mitochondrial fraction, since tumor-tissue homogenates behave in a fashion similar to liver.

Table 2 summarizes the effects on 4 enzymes produced either by physical disruption of cell particulates or by injection of derivatives of podophyllotoxin. The data on disruption of particulates are taken from Hogeboom and Schneider; the data on changes produced by NCI-3022 are our own, presented in this paper and in a previous one (*2*). It is apparent that the drug-induced changes in the cytochrome oxidase and in succinoxidase activity are more extensive than those produced by physical means, but that the changes produced in treated tissues on glutamic dehydrogenase and uricase activity are in no way comparable to those produced by physical means.

TABLE 2.—*Comparison of the effects on enzyme activities produced by the disruption of mitochondria by mechanical means and by the action of the chemical agents*

Enzyme	Percent of original activity after mechanical disruption of mitochondria*	Percent residual activity in tumor homogenates from mice, 24 hours after the treatment with NCI-3022
Cytochrome oxidase	73	66
Succinoxidase	50	2
Uricase	95	2
Glutamic dehydrogenase	700 (approx.)	46

*Data collected from Hogeboom and Schneider (*4, 5, 8, 9*).

These inferences are fortified by the results of Freeman, Saslaw, and Leiter (*11*) from this laboratory, who showed that when homogenates of Hepatoma 129 from animals treated with NCI-3022 were fractionated by differential centrifugation, no loss in mitochondrial nitrogen was observed despite the fact that only 15 percent of the cytochrome oxidase activity remained.

Adenosine deaminase and nucleoside phosphorylase activities were not so markedly affected as were mitochondrial enzymes such as succinoxidase or uricase. Although these are both concentrated in the nonsedimentable portion of the cell, it should not be presumed that the principal changes following drug treatment occur exclusively in the mitochondrial portion. Freeman *et al.* (*11*) have observed about a 35-percent decrease of RNA in homogenates from Hepatoma 129.

Summary

1) Homogenates of Sarcoma 37 from animals injected with 20 μg./gm. of acetylpodophyllotoxin-ω-pyridinium chloride showed a marked decrease in the mitochondrial enzyme uricase. Only 25 percent of the uricase activity of control tissue was observed in the tumor tissue from animals sacrificed 6 hours after injection of the drug.

2) Adenosine deaminase and nucleoside phosphorylase, two enzymes in the soluble fraction of the cell, were only slightly affected during the same time interval.

3) Smaller decreases were also observed in the glutamic dehydrogenase activity in homogenates of Hepatoma 129 from treated animals as compared with homogenates from untreated animals, even after the homogenates were forced through a tissue press at 20,000 pounds per square inch.

4) The glutamic dehydrogenase activity of a variety of tumors in untreated animals was determined.

5) The relationships between the biochemical changes and the structural integrity of some cell particulates are discussed.

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Hepatomas in Mice. I. Factors Affecting the Rapid Induction of a High Incidence of Hepatomas by *o*-Aminoazotoluene[1]

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In order to study the succession of morphologic and biochemical events leading up to the appearance of a grossly recognizable tumor, it would be desirable to have a situation in which these events occurred in one type of cell during a comparatively short interval of time. The cells of the liver are admirably suited for such a purpose, since they are available in quantity and are easily obtained for both biochemical and morphologic study. A high percentage of liver tumors can be induced rapidly in rats by feeding any of a variety of azo dyes (*1*), but the complexity of the alterations produced prior to the actual appearance of the tumors (*2*) and the uncertainty among pathologists as to what cell types give rise to the tumors (*2*) make the rat liver unsatisfactory for such a study. In the liver of the mouse, the morphologic alterations induced by the injection or feeding of azo dyes are comparatively uncomplicated, and the tumors that are produced are composed of cells remarkably like those of the hepatic parenchyma (*3*).[3] In the past, the use of the mouse in the study of hepatic carcinogenesis was found to be inexpedient because of the length of time required for the tumors to appear, the high mortality due to the toxicity of the carcinogenic dyes, and the low yield of tumors (*5*). The work of Andervont and his collaborators (*6–9*) has shown, however, that in inbred strains of mice the susceptibility to the induction of liver tumors by *o*-aminoazotoluene (4-amino-2,3-dimethylazobenzene) is conditioned largely by sex and strain and that the most susceptible animal is the strain A female. Strain A males are relatively resistant to hepatic-tumor formation. Although the susceptibility of this strain of mouse to the carcinogenic effects of *o*-aminoazotoluene is high, the incidence of spontaneous hepatomas is under 2 percent in mice well over a year of age. Therefore, the strain A female mouse was selected for the experiments to be reported in this paper.

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[1] Received for publication January 7, 1955.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] Kirby (*4*) reports finding a cholangioma in the liver of a female mouse dying at 425 days after having received a total of 130 mg. of *N,N*-dimethyl-*p*-aminoazobenzene.

These experiments were designed to provide the optimal conditions for producing a high yield of liver tumors in a short period of time. In addition to using a mouse of susceptible strain and sex, hormonal and dietary factors which were known to be of importance were also taken into consideration in planning the procedures.

Materials and Methods

Animals.—Strain A female mice of the Heston subline were used, except on a few occasions when an F_1 cross between 2 strain A sublines was tested. The female in this cross was of a subline carried for years in the research laboratories of Eli Lilly and Co. It is designated in this laboratory as A/LN. The male was of the Heston subline and is designated as A/HeN. Since the Lilly strain A is a better breeder than the Heston strain A, the hybrid was used in the hope of getting a more vigorous animal.

The mice were kept in plastic cages containing a wire-screen floor elevated above sawdust. The diets and water were fed *ad libitum*.

Diet.—The mice were fed a semisynthetic diet based upon one reported by Rusch *et al.* (*5*). The composition of the diet was as follows:

	(*percent*)
Casein (Labco Vitamin Free, the Borden Co.)	12
Dextrose (Cerelose, Corn Products Sales Co.)	64
Salt (GBI Salt Mix #2 USP XIII)	4
Corn oil (Mazola, Corn Products Refining Co.)	4.5
Lard (Swiftning, Swift and Co.)	15
Cod-liver oil (American Pharmaceutical Co.)	4.5

Crystalline vitamins (Nutritional Biochemicals Co.) as follows:

	(*mg./kg. of diet*)
Thiamine hydrochloride	7.0
Calcium pantothenate	20.0
Pyridoxine hydrochloride	4.5
Folic acid	5.0
Biotin	1.0
Choline chloride	30.0
Riboflavine (low riboflavine diet)	2.0
Riboflavine (high riboflavine diet)	10.0

Roughage in the form of an inert cellophane product (Spangles, Rayon Processing Co. of Rhode Island) was added to the diet at the level of 3 percent.

The dye.—*o*-Aminoazotoluene (Eastman Kodak Co.) was recrystallized from ethyl alcohol in distilled water and analyzed: C=74.40%, H=7.0%, N=18.79%, M.P.=104° C. The dye was dissolved in the corn oil and mixed with the diet at the level of 0.05 percent.

Stilbestrol.—One part of diethylstilbestrol (Merck) was combined, by melting in a hot oil bath, with 9 parts of cholesterol (Eastman Kodak Co.), and from this mixture 2 to 2.5 mg. pellets were prepared according

to the method of Shimkin and White (*10*). The stilbestrol pellets were implanted in the right axilla of the mice by means of a 13-gauge trocar.

Preparation of tissues.—Slices of liver about 2 mm. thick were fixed in Orth's fluid and in 10-percent buffered neutral formalin. The tissues in Orth's fluid were stored at 5° C. for 24 hours, rinsed in tap water, placed in 3-percent potassium bichromate at 37° C. for 24 hours, washed 18 to 24 hours in running water, dehydrated, and embedded in paraffin. Sections were cut at 3 and 4 microns and stained with eosin-azure for basophilia, aniline-acid fuchsin-methyl green for mitochondria, Laidlaw's stain for reticulum, and the periodic acid-Schiff reaction for glycogen. Tissues fixed in formalin were frozen, sectioned at 15 microns, and stained with Oil Red O for fat.

At the time of autopsy, slices of liver were frozen in dry ice, and later in the same day the tetrazolium reaction (using neotetrazolium) for succinic dehydrogenase (*11*) was run on frozen sections cut from the slices.

Design of experiments.—In all the feeding experiments, the mice were routinely fed the basal diet for two weeks before being placed on the dye diet.

Experiment 1.—This was designed to test 1) the effect of stilbestrol and 2) the duration of dye feeding upon the induction of tumors by *o*-aminoazotoluene.

Ninety-six mice, ranging in age from 3 to 5 months, were divided into groups of about 12 per cage. A stilbestrol pellet was injected into the right axilla of each animal. Twenty-seven animals that served as controls were fed the basal diet without the dye for the duration of the experiment. The remaining 69 mice were fed the diet containing 0.05-percent *o*-aminoazotoluene. At the end of 30, 60, and 90 days, groups of mice were removed from the dye diet and placed on the basal diet for the remainder of the experiment, which was of 120 days' duration. One group of mice was fed the dye diet for the entire 120 days.

Another group of 60 mice, ranging from 2 to 3 months of age, was placed on the dye diet. No stilbestrol pellets were injected. Half of these mice were removed from the dye regimen and placed on the basal diet after 88 days of feeding, while the remainder were fed the dye for a total of 115 days.

Twenty-six dye-fed mice and 7 control mice were found dead and were not autopsied because of extreme autolysis.

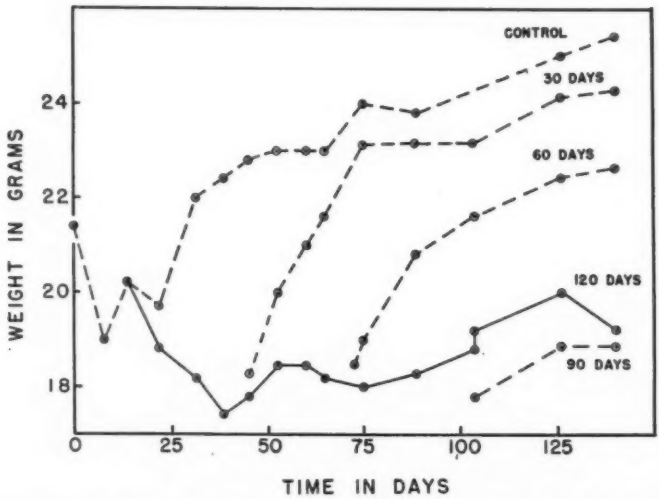
Experiment 2.—Serial killing—intermediate stages of tumor formation.—Forty-eight mice were injected with stilbestrol pellets and were placed on the dye diet. At intervals of 28, 57, and 91 days after the start of dye feeding, 12 mice were killed and autopsied. The mice killed after 91 days received the dye for 70 days and were returned to the basal diet for the remaining 21 days. The 7 mice remaining in the last group were placed on Purina laboratory chow. One of these mice was killed on the 128th day after the start of dye feeding, 2 mice were killed on the 160th day after the start of dye feeding, and 4 mice are still alive. Five mice died without autopsy during the first 2 months of dye feeding.

Experiment 3.—Effect of high riboflavine on tumor production.—Twenty-three mice with stilbestrol pellets were fed the dye diet containing 10 mg. of riboflavine per kilogram for 70 days and were returned to a high-riboflavine basal diet for 43 days, at which time all the mice were killed and autopsied. Four mice died without autopsy.

Experiment 4.—Production of tumors in hybrid mice.—Twenty-four hybrid mice with stilbestrol pellets were fed the dye for 70 days and then placed on the basal diet for an additional 42 days before all the animals were killed and autopsied. One mouse died without autopsy.

Results

A high percentage of the mice in all the feeding experiments survived the toxic effects of the dye. The basal diet supported growth, and the control mice appeared normal after eating the diet for 120 days. When the mice were placed on the dye diet, they immediately lost from 3 to 4 grams in weight and then maintained a weight of about 18 grams until they were returned to the basal diet. Text-figure 1 illustrates the typical relationship between average body weight and dye feeding.



TEXT-FIGURE 1.—Relation of body weight to dye feeding. The figure includes all the stilbestrol-treated mice in experiment 1. Each point represents the average weight of not less than 9 mice. The *top broken line* indicates the weight variation in the control mice. The *solid line* represents the weights of the mice eating the dye-containing diet. The *broken lines*, appearing at intervals above the solid line, represent the weights of the mice that were returned to the basal diet. The number of days that each group of animals was fed the dye is indicated at the end of the line.

Note the drop in weight and subsequent recovery during the first two weeks on the basal diet. At 90 days of dye feeding, mice with the lowest weights were returned to the basal diet, since they might not have survived longer on the dye diet. This accounts for the low values on the dotted line and the high values on the solid line.

The effect of stilbestrol upon the incidence of tumors in the mice 120 days after the start of dye feeding is shown in table 1. It can be seen that when mice were injected with a stilbestrol pellet, ingestion of the dye for only 60 days was sufficient to produce the maximum effect of the carcinogen. Feeding the dye for longer intervals of time did not increase the final tumor incidence. In contrast to this, in mice with no hormonal stimulation, the maximum effect of the carcinogen was not produced even after 88 days of dye feeding. After 120 days of dye feeding, however, the influence of stilbestrol upon tumor incidence was no longer detectable, since the same number of tumors occurred in both groups of mice without regard to the presence or absence of the hormone.

TABLE 1.—*Summary of experiments**

Experiment No.	Days on dye diet	Days on basal diet	Stilbestrol pellet	Number of mice	Percent survival	Percent of survivors having liver tumors
1 Effect of duration of dye feeding and of stilbestrol	0 (control)	120	+	20	74	0
	30	90	+	9	—	44
	60	60	+	11	63	82
	90	30	+	10	—	80
	120	0	+	12	—	83
	88	27	−	24	75	29†
	115	0	−	22	—	82
2 Serial killing	28	0	+	12	—	17‡
	57	0	+	12	90	67
	70	21	+	12	—	100
3 High riboflavine	70	43	+	19	83	89
4 Hybrid mice	70	42	+	23	96	78

*All tumors in this tabulation were observed as macroscopic, well-demarcated nodules, except for (†) where 1 microscopic tumor was included and for (‡) where 2 microscopic tumors (see fig. 5) were counted.

Table 1 also gives a summary of experiments 2, 3, and 4. In examining the results of experiment 2 one can see that tumors begin to appear very early. Two very small tumors were observed microscopically after only 28 days. After 57 days, 8 out of 12 mice had grossly recognizable tumors, while all the mice killed after 90 days had multiple liver tumors.

With regard to experiment 3, it is apparent from the percentage of mice bearing tumors at 113 days after the start of the dye feeding on the *high riboflavine* diet, that high riboflavine does not protect against hepatic-tumor formation in the mouse. On the other hand, the strain of mouse does seem to be important. The hybrid mice (expt. 4) were less affected by the carcinogen than were the mice of strain A/HeN. Not only did fewer hybrid mice have tumors but in most cases only 1 or 2 tumors were observed grossly, while in a comparable time interval in the Heston subline of strain A it was the rule to find multiple large tumors involving more than one lobe of the liver.

The tumors appeared grossly as circumscribed nodules protruding from the surface of the liver. They varied in size from barely visible nodules 1 mm. in diameter to large tumors 1.5 cm. in diameter. They were usually pale pink or tan in color and clearly demarcated from the dark red of the normal liver tissue (fig. 1). Many showed prominent blood vessels on the surface.

During the course of these experiments a record was kept of the distribution of the tumors among the various liver lobes (table 2). No attempt was made to count the total number of tumors in each lobe since the purpose of the enumeration was to determine in which lobe of the liver the tumors most frequently occurred. In addition to the readily recognizable macroscopic tumors observed at autopsy, 3 microscopic tumors (such as shown in fig. 5) were also included in the tabulation. Out of a total of 57 mice bearing tumors, the tumors occurred in the left lateral lobe 47 times, or in 82 percent of the animals. Thus, samples of tissue taken from the left lateral lobe of the liver during the period of dye feeding would most likely yield information as to the changes taking place prior to the appearance of the tumor.

TABLE 2.—*Tabulation of the distribution and the number of times gross tumors were found in the various lobes of the liver*

Group	Left lateral	Median	Quadrate	Caudate
Serial killings	19	18	16	8
High riboflavine	16	13	14	8
Hybrid	12	6	11	5
Total	47	37	41	21
TOTAL MICE=57				

Microscopic observations.—A paper by Andervont, Grady, and Edwards (*6*) describes the changes observed in the liver when the mice were injected subcutaneously with *o*-aminoazotoluene and allowed to feed *ad libitum* on a commercial chow diet. In mice so treated, no macroscopic hepatic alterations were observed until 32 weeks after the first injection of the dye, and tumors did not appear until even later. The characteristics of hepatomas in mice have been described in detail by Andervont and Dunn (*3*).

The nature of the histopathologic changes which appeared in the livers of the mice in the present experiments were similar to those described by Andervont, Grady, and Edwards (*6*), but the alterations were less pronounced and occurred in a much shorter period of time. It was not possible to produce exactly the same degree of alteration in the mouse liver in the different feeding experiments, although the general features of the changes were the same. Thus the livers of the stilbestrol-treated mice in the first experiment appeared to show more extensive proliferation of the nonparenchymal cells and more aberration in the size and shape of the parenchymal cells than was observed in the livers of the mice in the second experiment (serial killing). Conversely, more tumors appeared

in the livers of mice in the second experiment than in the livers of mice in the first experiment. There was also some indication that the alterations initiated by the dye continued to progress even after the mice were removed from the dye and placed on the basal diet. Thus the livers of mice fed the dye for 30 days and the basal diet for 90 days (expt. 1) showed more extensive histopathologic changes than did the livers of mice killed after being fed the dye for a total of 60 days (expt. 2).

In order to obtain an understanding of the progression of the changes occurring in the livers of mice fed *o*-aminoazotoluene, a series of animals were killed at about 4-week intervals after the start of dye feeding (expt. 2). The hepatic alterations were remarkably uniform within a group of mice killed at the same time. Around the portal areas of the livers after only 4 weeks of feeding there was an increase in the number and prominence of the endothelial cells, an increase in reticular fibers, and some evidence of bile-duct proliferation (figs. 3 and 4). The central area of the lobules appeared unchanged. There was a heavy, uniform storage of glycogen in the parenchymal cells. This heavy storage of glycogen was also characteristic of the livers of the control mice. In the livers of 2 mice at this 4-week interval, two circular nodules about 0.3 mm. in diameter were found next to a central vein. In morphology the cells of these nodules appeared to be similar to those of the adjacent parenchyma, but they had lost their orientation with respect to the normal architecture of the liver. They grew in a concentric pattern and compressed many of the contiguous liver cells (fig. 5). Some of the cells in the nodules were in mitosis. Most of the cells of one of the nodules were free of glycogen, while the cells in the other nodule contained about as much as the surrounding liver. These small groups of cells were clearly demarcated from the normal parenchyma of the liver, and it is probable that they represented the earliest focus of a hepatoma.

As the period of dye feeding was prolonged, most of the parenchymal cells increased in size, and some of them became enormously swollen and distorted in shape. Frequently the nuclei of these cells were enlarged and distorted, and some contained intranuclear inclusions (figs. 6 and 7) which took an intense stain after periodic acid-Schiff treatment without diastase but were unstained if diastase digestion was done before treatment. This indicated that these bodies contained glycogen. The orderly arrangement of the hepatic cells was disrupted by the progressive increase in reticulum (fig. 8) and in the number of cells differing morphologically from those of the hepatic parenchyma. Only very mild fibrosis was observed in the livers of mice killed after 90 days in experiment 2, whereas a more marked cellular proliferation occurred in the livers of mice fed the dye for 120 days (expt. 1). In the livers of the latter animals, strands or sheets of cells with elongated nuclei, together with cuboidal cells forming duct-like structures, extended from the portal veins for some distance into the interlobular spaces and separated the parenchymal cells from each other. Large cells that appeared to be undergoing degeneration were frequently found in the lumina formed by the sur-

rounding cuboidal cells (fig. 9). In some livers, at this time, there were circular areas up to 1 mm. in diameter composed of tortuous, dilated spaces lined with cuboidal cells (fig. 10), but in most of the mice at all stages of dye feeding these dilatations were present to a very limited extent. The livers of the mice receiving no stilbestrol were characterized by an even more pronounced fibrosis. When cut at autopsy, these livers were firm and tough, and it was observed microscopically that the amount of reticular fibers and fibrous tissue was greater than in the livers of the stilbestrol-treated animals.

When gross tumors were present (which was as early as 57 days after the beginning of dye feeding), microscopic areas suggestive of beginning neoplasms were also found occasionally. These were often composed of cells of uniform size, usually somewhat smaller than the cells of the surrounding liver. The more advanced tumors, found in mice killed at the 160-day interval, often contained a central core of enlarged cells which compressed adjacent areas of small tumor cells, forming a "tumor within a tumor" and suggesting that a further anaplastic alteration had taken place in some of the cells. Glycogen was more often absent from the tumors than present, whereas fat was almost always present (fig. 11). Fat was observed less frequently and was less abundant within the cells of the surrounding nontumorous liver. The distribution of fat in the tumors did not follow any consistent pattern; it could be either closely associated with the blood vessels or present in cells located at random throughout the tumor. The cells of the tumors were markedly basophilic as compared with the cells of the surrounding liver. Mitochondria of many different sizes and shapes were seen in various tumors, but in general they tended to be short, fat rods which exhibited no variation in shape relative to their proximity to the blood vessels. The tumors contained very little fibrous tissue (fig. 12). They could be shelled out from the liver and were usually soft in consistency.

The capacity of slices of liver containing tumors to reduce neotetrazolium was tested in livers from 30 different mice killed at intervals of from 57 to 120 days after the beginning of dye feeding. In every case, the tumor cells contained more reduced tetrazolium than did the cells of the adjacent liver (fig. 2).

Discussion

The work reported here was initiated in the hope that tumors composed predominantly of hepatic parenchymal cells could be produced in a high percentage of experimental animals in a short period of time, since this would provide an opportunity to examine by morphologic and chemical means the changes that take place in these cells during their transformation from normal to tumor cells. This was in part accomplished by utilizing the observation of Andervont (*8*, *9*) that the susceptibility of mice to the carcinogenic action of *o*-aminoazotoluene depended upon their strain and sex. The present studies show that if female strain A mice are given, in addition to the dye, a high dose of artificial female sex

hormone (stilbestrol), tumors will appear within a few weeks in a high percentage of animals.

The mode of action of stilbestrol in enhancing the action of *o*-aminoazotoluene is not known, nor have the dietary factors related to the development of tumors in mice by *o*-aminoazotoluene been thoroughly investigated. The fact that riboflavine does not protect the animals from the action of the carcinogen indicates that this vitamin is less important to the mouse in this regard than it is to the rat (*12*).

The results of the histochemical test for succinic dehydrogenase by the tetrazolium method were surprising. If the tetrazolium reaction is indeed a measure of succinic dehydrogenase activity, then the activity of the enzyme in these tumors is substantially greater than its activity in the surrounding liver. This is in direct contrast to the situation in rat and mouse hepatomas as reported by Potter *et al.* (*13*) and Schneider and Hogeboom (*14*), who found that the succinic dehydrogenase activity of azo dye-induced rat-liver tumors and of a transplantable spontaneous mouse hepatoma was less than that of the normal liver. A direct chemical estimate of the succinic dehydrogenase activity of these mouse-liver tumors is needed in order to ascertain the true interpretation of the histochemical results.

The question of whether or not the small nodules of hepatic cells observed microscopically after 28 days of dye feeding represent neoplasia or areas of hyperplasia is an important one. The formation of a tumor is a dynamic process that must have a beginning as well as an end. A full-blown tumor is readily recognized, but the criteria by which one determines a very early tumor in the liver have not been generally established. The small nodules in question were distinctly different from the surrounding liver parenchyma. In fact, at that interval of dye feeding, these areas were the only areas involving hepatic cells which were clearly separated from the normal parenchyma. The changes found in the liver at the time the nodules appeared were not associated with the parenchymal cells but involved the stromal elements and bile ducts; neither of these groups of cells was implicated in the formation of the final tumors. It seems probable, therefore, that the nodules composed of hepatic parenchymal cells represented a true neoplastic alteration which, if allowed to proceed, would have resulted in unmistakable tumors.

Summary

Tumors of the hepatic parenchymal cells can be produced in 80 to 100 percent of strain A female mice by adding the stimulus of the female sex hormone, stilbestrol, to the carcinogenic action of the azo dye, *o*-aminoazotoluene. When the dye was fed in a semisynthetic diet it was found that:

1) In combination with stilbestrol, it was necessary to feed the dye for only 60 days in order to achieve a maximum carcinogenic effect.

2) Tumors began to appear as early as 28 days after the beginning of dye feeding, and in one experiment 100 percent of the animals had gross tumors after 90 days.

3) High riboflavine in the diet did not protect the mice against the carcinogenic action of the dye.

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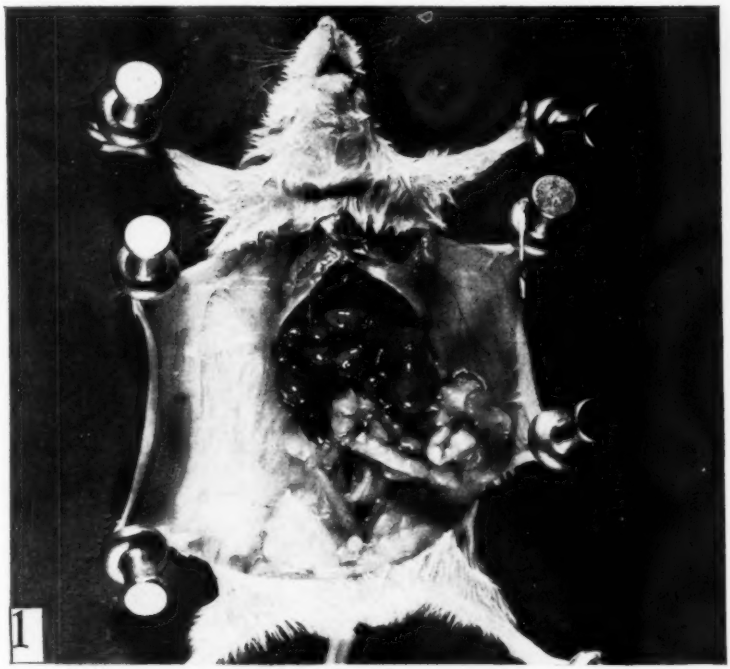
PLATES

PLATE 8

FIGURE 1.—Multiple tumors in the liver of a mouse fed *o*-aminoazotoluene for 90 days (expt. 1). Every lobe except the caudate is involved.

FIGURE 2.—Small tumor sharply demarcated from the surrounding liver by the relatively greater amount of reduced tetrazolium deposited in its cells [from a mouse killed after 57 days of dye feeding (expt. 2)]. × 45



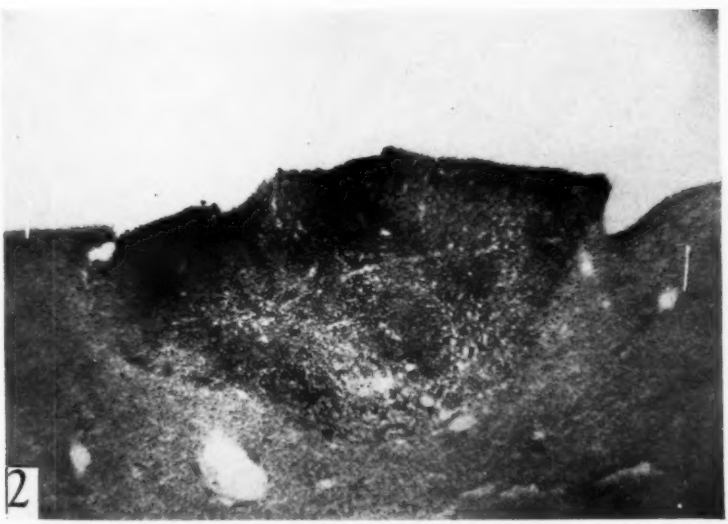


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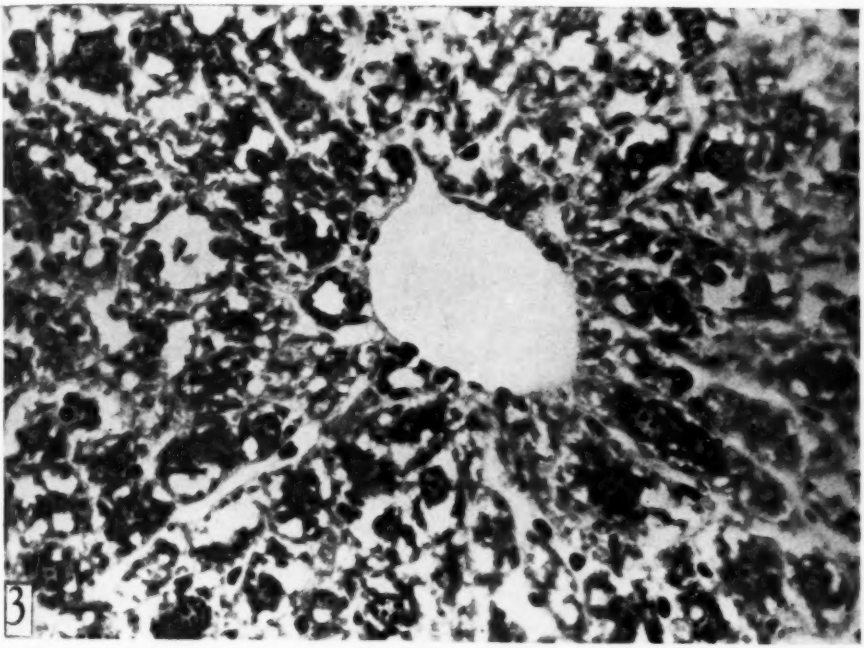
PLATE 9

FIGURE 3.—Portal area in liver of mouse fed the basal diet for 134 days. Shown for purposes of comparison with figure 4. Eosin-azure. × 550

FIGURE 4.—Portal area in liver of mouse fed *o*-aminoazotoluene for 28 days. Observe that the parenchymal cells are very similar to those in the untreated liver (fig. 3), but proliferation of the cells of the hepatic stroma can be seen around the vein. Eosin-azure. × 550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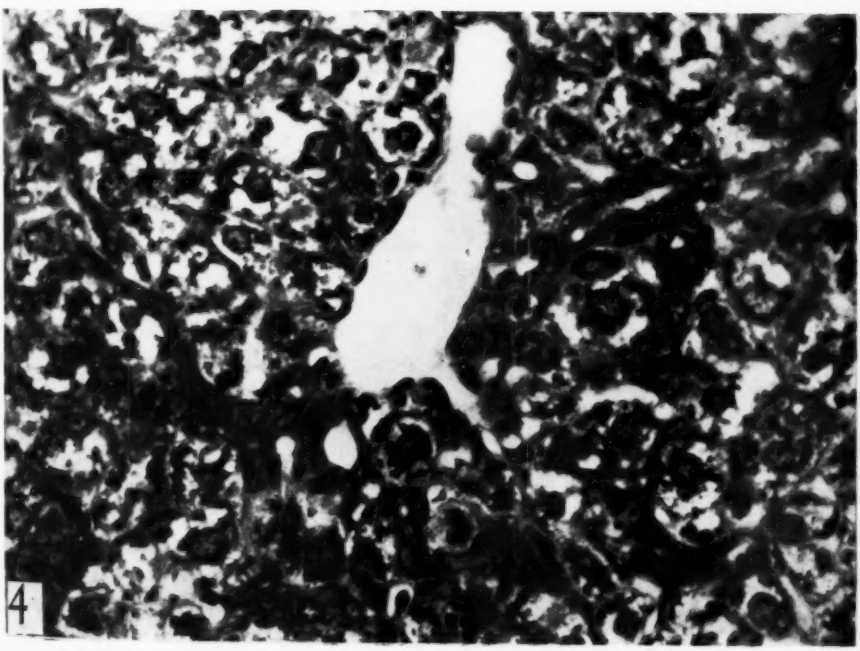
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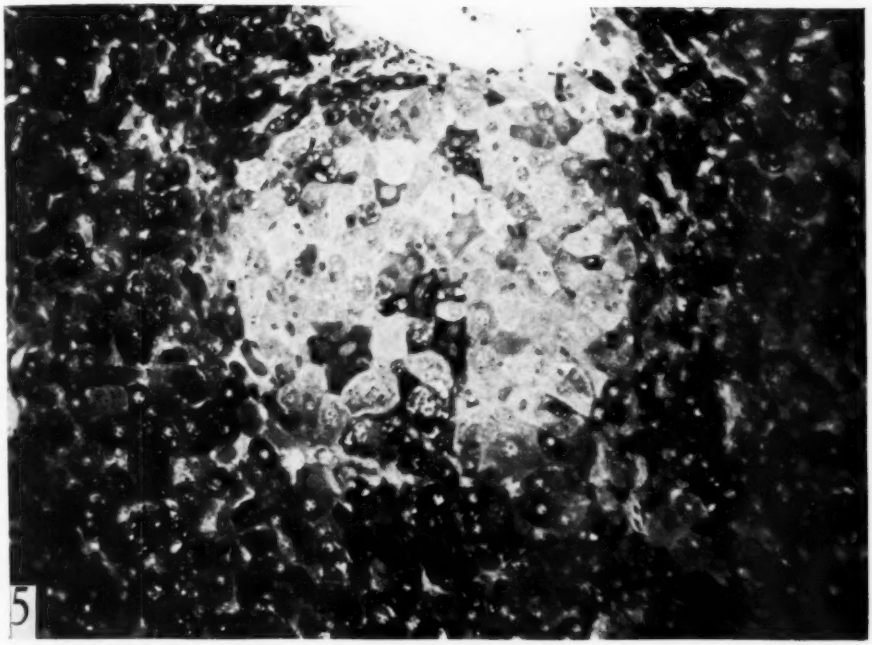
PLATE 10

FIGURE 5.—Small nodule of cells, 0.3 mm. in diamter, adjacent to a central vein in mouse fed *o*-aminoazotoluene for 28 days. Note the concentric arrangement of the cells in the nodule and the distortion of the contiguous cells. Cells in the nodule are generally free of glycogen. Periodic acid-Schiff. × 200

FIGURE 6.—Intranuclear inclusions of glycogen in cells from liver of mouse fed *o*-aminoazotoluene for 30 days (expt. 1)—compare with figure 5. Periodic acid-Schiff. × 500

FIGURE 7.—Adjacent section from same liver as shown in figure 6, treated with diastase prior to the periodic acid-Schiff reaction. Note that enzymatic digestion has removed the glycogen from the intranuclear inclusions leaving large unstained vacuoles. Periodic acid-Schiff. × 500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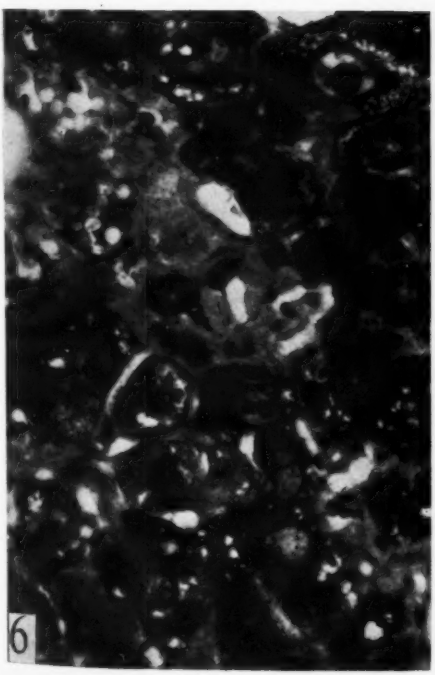
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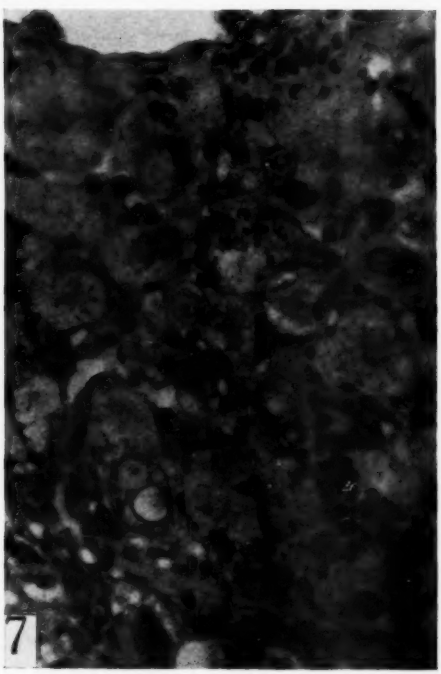
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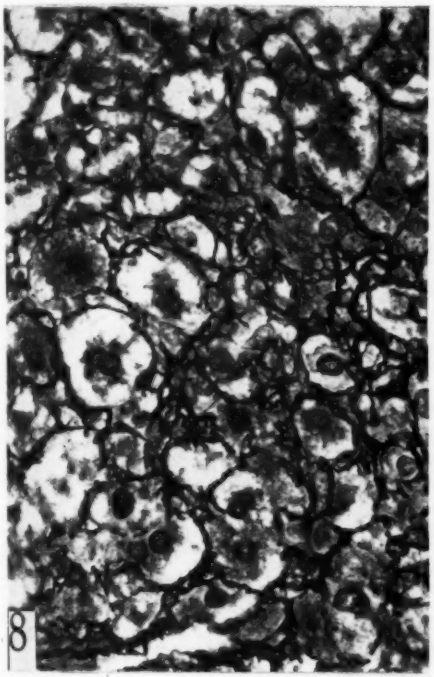
PLATE 11

FIGURE 8.—Reticulum stain on liver of mouse fed *o*-aminoazotoluene for 90 days (expt. 2). Many liver cells are enlarged and all are surrounded by a thick network of reticular fibers. × 500

FIGURE 9.—Proliferation of nonparenchymal cells in liver of mouse fed *o*-aminoazotoluene for 120 days (expt. 1). Large cells within the lumina of the duct-like structures lined by cuboidal cells appear to be undergoing degeneration. Eosin-azure. × 550

FIGURE 10.—Dilatations lined by cuboidal epithelium in the liver of a mouse fed *o*-aminoazotoluene for 120 days (expt. 1). Eosin-azure. × 550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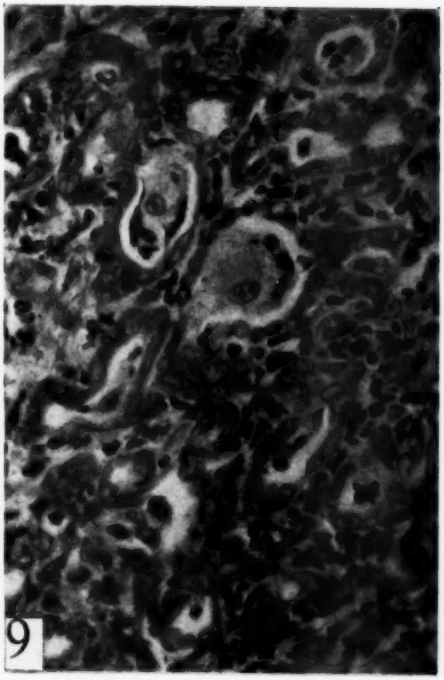
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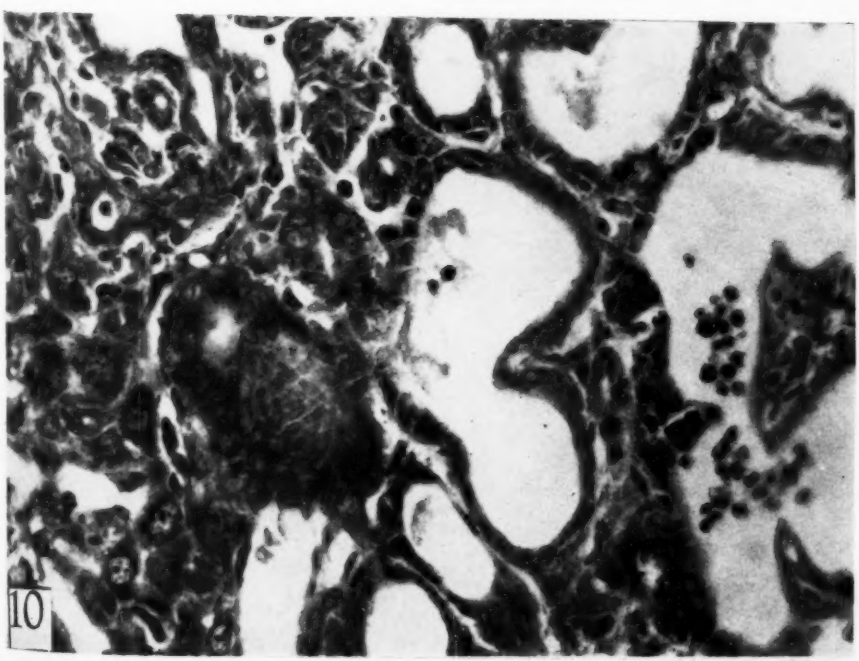
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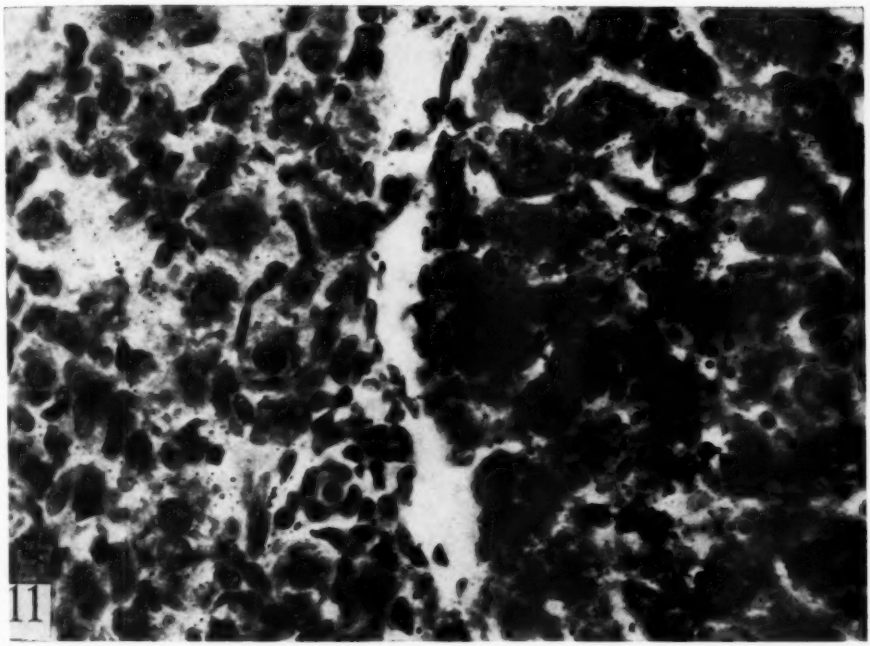
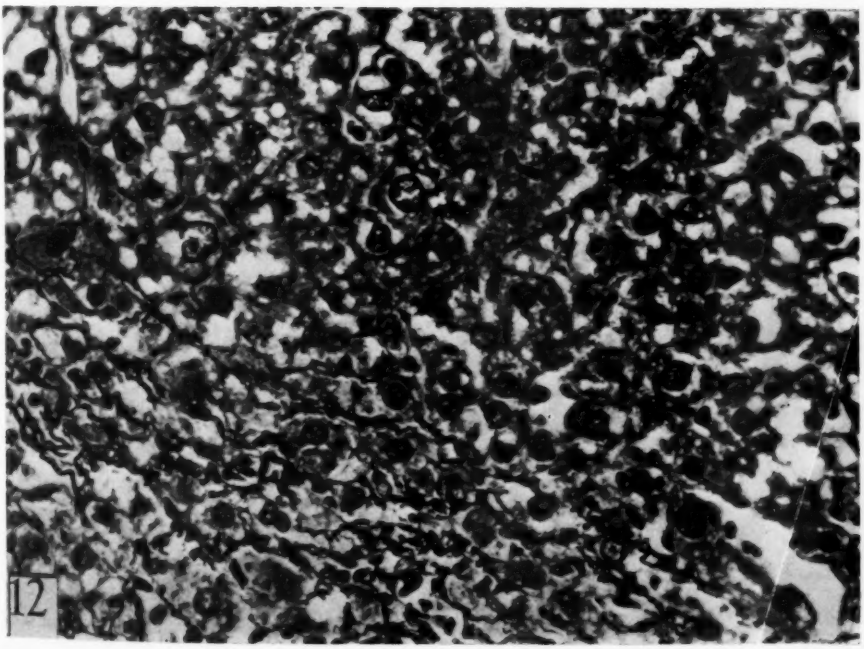
PLATE 12

FIGURE 11.—Edge of a tumor (*at right*) and adjacent liver separated by a vascular space. Many of the tumor cells are almost obscured by large fat droplets, while rather inconspicuous fat droplets are present in the non-neoplastic liver. Oil Red O for fat. × 500

FIGURE 12.—Edge of tumor in mouse fed *o*-aminoazotoluene for 90 days (expt. 2). Reticulum is lacking in the tumor, which is sharply demarcated from the adjacent liver. Note the small size of the tumor cells. Laidlaw's reticulum. × 500





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An Investigation into the Synergistic Antileukemic Action of Amethopterin and 6-Mercaptopurine in Mice[1]

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In recent years there has been increasing interest in the treatment of experimental tumors with combinations of drugs. The primary objective has been to determine whether the treatment of tumors with combinations of two or more drugs might prove to be more effective than treatment with a single drug (*1–14*). Combination drug chemotherapy has also been employed to investigate biochemical differences between tumor and host, and problems in sequential and concurrent blocking of enzymatic transformations (*10, 13, 15, 16*). It has been used in an effort to overcome the development of resistant and dependent variant tumor cells (*4, 5*). In general, the results of such studies have been related to the observed synergistic or nonsynergistic activity of the combination treatment employed.

Almost without exception, the emphasis in the combination drug chemotherapy studies has been on the effect of the treatment on the tumor, with little consideration of the relationship of host toxicity to anti-tumor activity of the drugs. As long as each drug was used at or below its maximum tolerated dose, it was felt safe to disregard host effects. However, this ignores the fact that the combination of the two drugs may be above the maximum tolerated dose, and, in any case, may well produce increased toxic effects on the host.

In this laboratory a macrobiological assay procedure has been developed, employing levels of treatment both above and below the maximum tolerated dose, for investigating the antileukemic specificity of action of various treatments (*17–19*). The antileukemic specificity of action is described in a quantitative manner in terms of the relative effect of the treatment with respect to host and tumor. Comparison of the specificities of antileukemic action of two treatments is made by comparing the effects of the two treatments on survival time at equal percent toxic mortality. In the present study, these methods are employed to investigate the antileukemic action of amethopterin and 6-mercaptopurine in com-

[1] Received for publication December 13, 1954.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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bination. Three major points relative to the use of combination treatment with the two drugs can be examined in the light of the present study. These are:

a) Is there any evidence for synergism with respect to effect on the leukemia alone, disregarding any effects on the host? The two drugs in a particular combination may have greater antileukemic action than does either drug employed separately, but this fact in itself is not evidence for synergism. It should further be required that the antileukemic effect of the combination be greater than the sum of the antileukemic effects of the drugs used separately.[3]

b) Is there any evidence of synergism with respect to effect on the host, disregarding any effects on the tumor? Even though the drugs may prove synergistic in their antileukemic action, this may be offset by synergistic toxic effects to the host. From a practical point of view, for therapy, it may be desirable to consider antileukemic synergism between the two drugs as existing only if, by using the two together, greater antileukemic damage can be accomplished at a fixed cost in toxicity to the host. If synergism exists in this sense, there should be a combination of amethopterin and 6-mercaptopurine such that for a fixed cost in host toxicity there is greater antileukemic damage than either of the drugs themselves would yield.

c) If the drugs are synergistic in the sense suggested in *b*), what are the optimal proportions in which they should be used to attain maximal antileukemic damage for fixed cost in toxicity to the host?

To answer these questions an experiment was conducted employing various proportions of amethopterin and 6-mercaptopurine, with graded doses at each proportion. The different combinations were: amethopterin alone; 7.5 parts amethopterin to 1 part 6-mercaptopurine; 1 part amethopterin to 1 part 6-mercaptopurine; 1 part amethopterin to 7.5 parts 6-mercaptopurine; 6-mercaptopurine alone.

The study permitted, incidentally, a comparison of the separate antileukemic specificities of amethopertin and 6-mercaptopurine as applied in the current experiments.

Methods

The experiment was conducted with leukemia L 1210 in 6- to 8-week-old male mice (BALB/cAn $\times$ DBA/2J)F_1. The techniques have been previously described (*17–19*). Stock tumor was taken from DBA/2J male mice. The animals were given tumor inoculations in the right thigh of 0.1 ml. of a uniform saline suspension of tumor cells containing 1.13×10^6 cells. Amethopterin[4] was prepared in 2-percent sodium bicarbonate at *p*H 7 to 8 and was administered to the mice in the constant volume of

[3] An alternative requirement might be that the antileukemic effect of the two drugs administered in combination exceed that corresponding to the dosage equivalent of the combination. In terms of drug A, the dosage equivalent of X units of drug A plus Y units of drug B is $X + Y^1$, where Y^1 units of A would elicit the same response as do Y units of B. This defines synergism relative to additivity of dose as opposed to synergism relative to additivity of effects.

[4] Amethopterin was kindly provided by Dr. J. M. Smith, Jr., of the Calco Chemical Division of the American Cyanamid Company, Bound Brook, N. J.

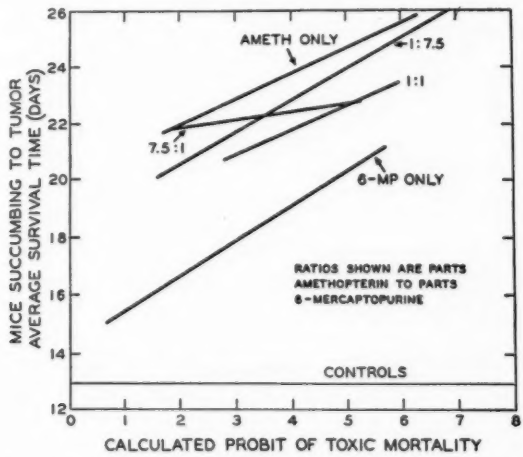
0.01 ml. per gm. 6-Mercaptopurine[5] was dissolved in dilute sodium hydroxide, and the solution was titrated back to *p*H 9 to 10 with dilute hydrochloric acid. The 6-mercaptopurine was administered in the constant volume of 0.03 ml. per gm., except at the doses of 1,600 mg. per kg. and 1,200 mg. per kg., where constant volumes of 0.055 ml. per gm and 0.04 ml. per gm., respectively, were given.

Both the mice and the order of injections were randomized. The amethopterin and 6-mercaptopurine were administered concomitantly. All injections were given intraperitoneally. The drugs were injected 3 days following tumor inoculation. The doses employed are listed in the experiment. As previously described (*17–19*), mice that succumbed to drug toxicity and mice that succumbed to tumor growth were separated on a temporal basis.

Results

The results of the experiment are presented in text-figures 1 to 4, the supporting data appearing in table 1.

In text-figure 1 are shown the fitted lines relating the average survival time for mice succumbing to tumor to the probit of toxic mortality resulting from treatment for the various treatments employed. It is seen that for given cost in toxic mortality, there is a longer survival time for mice treated with amethopterin alone and a shorter survival time for the mice treated with 6-mercaptopurine alone. The three combination treatments with amethopterin plus 6-mercaptopurine fall between



TEXT-FIGURE 1.—(See table 1.) Effect of combination treatment with amethopterin and 6-mercaptopurine on the survival time-toxic mortality relationship of leukemic mice. Fitted least squares relationships between time to death from tumor for mice surviving drug(s) toxicity and the calculated probit of toxic mortality.

[5] 6-Mercaptopurine was kindly provided by Dr. George Hitchings of the Wellcome Research Laboratories, Tuckahoe 7, N. Y.

TABLE 1.—*Toxicity and survival times for leukemic mice treated with amethopterin and 6-mercaptopurine**

(D=deaths among 8 mice from drug toxicity; *P*=calculated probit of toxic mortality†; S. T./N=average survival time in days for mice surviving drug toxicity but succumbing to tumor/number of mice on which this average is based)

Amethopterin only				Amethopterin + 6-mercaptopurine (7.5:1)					Amethopterin + 6-mercaptopurine (1:1)			
Amethopterin (*mg./kg.*)	D	*P*	S. T./N	Amethopterin (*mg./kg.*)	6-Mercaptopurine (*mg./kg.*)	D	*P*	S. T./N	Dosage of each (*mg./kg.*)	D	*P*	S. T./N
38	0	1. 69	22. 4/8	38	5. 1	0	1. 89	21. 9/8	28	1	2. 80	21. 1/7
50	0	2. 26	19. 3/8	50	6. 7	0	2. 44	22. 5/8	38	1	3. 24	20. 6/7
67	1	2. 82	23. 3/7	67	9	0	3. 00	22. 0/8	50	2	3. 68	21. 8/6
90	0	3. 38	26. 8/8	90	12	1	3. 55	21. 0/6‡	67	0	4. 12	22. 1/8
120	1	3. 94	21. 0/7	120	16	2	4. 10	22. 2/6	90	0	4. 56	21. 4/8
160	1	4. 51	26. 0/7	160	21	2	4. 65	24. 3/6	120	4	5. 00	23. 2/4
213	6	5. 07	19. 0/1‡	213	28	5	5. 21	22. 3/3	160	5	5. 44	24. 7/3
284	5	5. 63	26. 3/3	284	38	6	5. 76	22. 0/2	213	7	5. 88	21. 0/1
379	7	6. 20	20. 0/1	379	50	7	6. 31	‡	284	8	6. 32	
506	8	6. 76		506	67	8	6. 87		379	8	6. 76	
675	8	7. 32		675	90	8	7. 42		506	8	7. 20	
900	8	7. 88		900	120	8	7. 97		675	8	7. 64	

6-Mercaptopurine only				Amethopterin + 6-mercaptopurine (1:7.5)				
6-Mercaptopurine (*mg./kg.*)	D	*P*	S. T./N	Amethopterin (*mg./kg.*)	6-Mercaptopurine (*mg./kg.*)	D	*P*	S. T./N
90	0	0. 57	15. 2/8	9	67	0	1. 60	19. 6/8
120	0	1. 21	15. 2/8	12	90	0	2. 40	20. 9/8
160	0	1. 86	16. 1/8	16	120	0	3. 20	22. 6/8
213	0	2. 50	17. 6/8	21	160	1	4. 00	23. 9/7
284	0	3. 15	17. 9/8	28	213	4	4. 80	22. 2/4
379	2	3. 79	19. 7/6	38	284	7	5. 60	25. 0/1
506	3	4. 44	20. 6/5	50	379	7	6. 40	27. 0/1
675	2	5. 08	19. 3/6	67	506	7	7. 20	24. 0/1
900	6	5. 73	21. 5/2	90	675	8	8. 00	
1, 200	8	6. 37		120	900	8	8. 80	
1, 600	8	7. 02		160	1, 200	8	9. 60	

Controls: 48 mice in various control groups (2-percent $NaHCO_3$; untreated) all died of tumor between the 11th and 17th days following tumor inoculation; average = 12.94 days.

*(BALB/cAn × DBA/2J)F_1 male mice; stock tumor from DBA/2J male mice; tumor inoculum 1.13×10^6 cells per mouse; treatment 3 days following tumor implantation. In a titration of the tumor, 100-percent mortality was achieved with 1.13×10^4 cells per mouse.

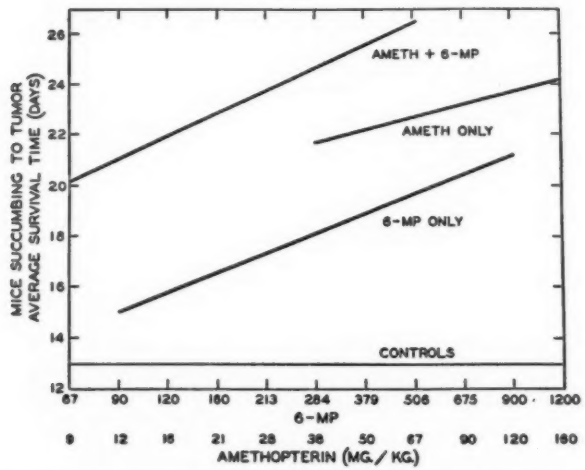
†Calculated probits obtained using the Cornfield-Mantel adaptation of Kärber's method (*20*).

‡Excludes 1 late death. Animal died without weight loss or evidence of local tumor.

these two extremes. This result is interpreted as meaning that, with respect to antileukemic specificity of action, there is no synergism between amethopterin and 6-mercaptopurine.

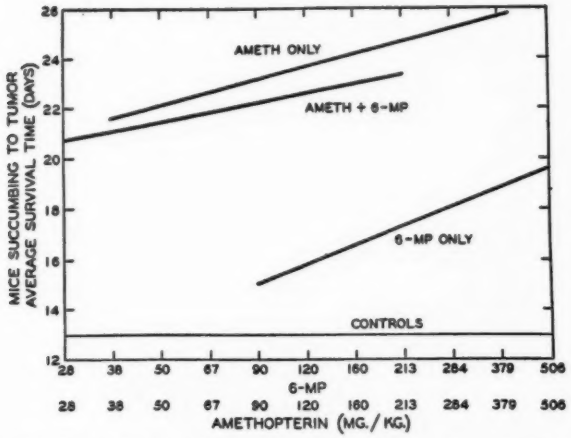
In text-figures 2 and 3 are shown fitted lines relating average survival time to dose for various treatments. These figures permit evaluation of effects of treatment with respect to the tumor without regard to effects on the host. The drugs administered shown in text-figure 2 are 1 part amethopterin to 7.5 parts 6-mercaptopurine, amethopterin alone, and 6-mercaptopurine alone. It is clear from this chart that in the amethopterin +6-mercaptopurine (1:7.5) combination the two drugs yield a greater survival time than does either of the drugs separately. This is similar to the result reported by Skipper *et al.* (*14*). Presumably the drugs in combination do greater antileukemic damage than does either separately. However, when one compares the increase in survival time over the control survival time yielded by the drugs in combination with the sum of the separate increases yielded by the two drugs, there is no clear evidence of synergistic antileukemic activity.[6]

Text-figure 3 presents the corresponding results for the 1 part amethopterin to 1 part 6-mercaptopurine combination. In this combination, the two drugs together do not seem to do more antileukemic damage than does the amethopterin component separately. The closeness of the amethopterin = only line to the amethopterin + 6-mercaptopurine (1:1) combination line suggests that, in the absence of synergism, the levels of



TEXT-FIGURE 2.—(See table 1.) Effect of combination treatment with amethopterin and 6-mercaptopurine on the survival time-drug dosage relationship in leukemic mice. Fitted least squares relationships between time to death from tumor for mice surviving drug(s) toxicity and the logarithm of the dose of drug(s) are plotted. Ratio of amethopterin to 6-mercaptopurine in the combination treatment is 1 to 7.5.

[6] With respect to additivity of dosage (see footnote 3), text-figure 2 does suggest synergism relative to effects on the tumor only. This synergism is no longer evident when increased effects on the host are also considered.

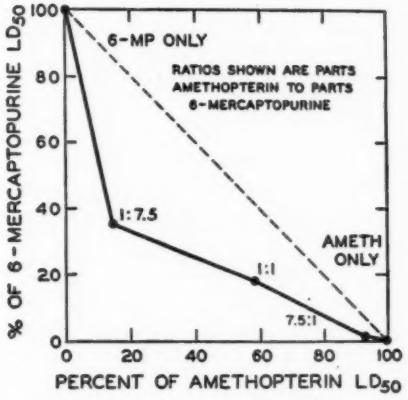


TEXT-FIGURE 3.—(See table 1.) Effect of combination treatment with amethopterin and 6-mercaptopurine on the survival time-drug dosage relationship in leukemic mice. Fitted least squares relationships between time to death from tumor for mice surviving drug(s) toxicity and the logarithm of the dose of drug(s) are plotted. Ratio of amethopterin to 6-mercaptopurine in the combination treatment is 1 to 1.

6-mercaptopurine employed were too low to enhance the antileukemic effect of amethopterin appreciably.

No text-figure similar to 2 and 3 is shown for the 7.5 parts amethopterin to 1 part 6-mercaptopurine combination. In these proportions the levels of 6-mercaptopurine used, in combinations not 100-percent lethal, were too small to produce significant toxic or antileukemic effects when administered alone.

Text-figure 4 shows, for each of the various proportions used, the median lethal combination (LD_{50}) of the two drugs. In each case the LD_{50} of



TEXT-FIGURE 4.—(See table 1.) Comparison of host toxicities of amethopterin and 6-mercaptopurine in combination treatment in leukemic mice. In each case the LD_{50} of the combination is expressed as percentages of the LD_{50}'s of the two drugs employed separately.

the combination is expressed as percentages of the LD_{50}'s of the two drugs employed separately. In each case where the two drugs were used in combination, the sum of the percentages of the separate LD_{50}'s associated with the combination LD_{50} is less than 100. For example, in the 1 part amethopterin to 7.5 parts 6-mercaptopurine combination, the LD_{50} is 30 mg. per kg. amethopterin + 229 mg. per kg. 6-mercaptopurine. This is 15 percent of the separate amethopterin LD_{50} + 35 percent of the separate 6-mercaptopurine LD_{50}, for a total of 50 percent. This would suggest that the two drugs are synergistic with respect to host toxicity.[7]

Discussion

In the current investigation, no enhancement in antileukemic specificity of action was obtained by combination treatment with amethopterin and 6-mercaptopurine on a single day. In the sense of increased antileukemic effects for fixed host toxicity, the two drugs were not synergistic.

Doubt is also cast on whether the two drugs evidenced synergism with respect to their antileukemic action, disregarding any effect on the host. When used together, there was increased antileukemic effect as compared with the separate effects produced by the drugs. However, the increased effect of the combination treatment was not clearly more than the sum of the separate effects. It is clear, however, that whatever synergism there may have been with respect to antileukemic damage, it was offset by a comparable synergism with respect to toxicity to the mouse.

It would be of interest to determine whether synergism between these two drugs could be evoked through modification of treatment schedules. Examples of the effects of modifying schedules of treatment in combination therapy are provided by the authors' investigations (*17*, *18*) with aminopterin and citrovorum factor. The use of aminopterin and citrovorum factor simultaneously resulted in a lowering of the antileukemic specificity of action. By delaying the administration of citrovorum factor for 12 to 24 hours, the antileukemic specificity of action was increased.

An incidental result of the study is the finding that, on single application as employed, amethopterin showed greater antileukemic specificity than 6-mercaptopurine.

These experiments emphasize further the importance of a consideration of the host-tumor relationship in determining the effectiveness of antitumor chemotherapeutic agents, whether administered individually or in combination. The macrobiological assay procedure employed here and previously (*17–19*) may be applied to *in vivo* studies of phenomena such as tumor resistance and dependence. It may also be of use in helping to uncover relations between metabolites and antimetabolites, and in the elucidation of the nature of metabolic transformations.

[7] While results such as these are ordinarily taken to indicate synergism, the authors feel that under certain circumstances, similar results can be obtained in nonsynergistic cases.

Summary

The authors have suggested that, in an investigation of synergism between two drugs, one should consider what detrimental effects the two drugs produce simultaneously with their enhanced beneficial effects. With this point of view, an experiment was conducted in which mice with a transplanted mouse leukemia were treated concomitantly with single doses of amethopterin and 6-mercaptopurine. Combination treatment with these drugs had previously been reported as synergistic. In the present study, however, it was found that the combination treatment resulted in such increased detrimental effects in terms of host toxicity that the two drugs together did not provide a synergistic therapy.

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The Effect of Hypophysectomy and X Irradiation on Lymphoid Organs and on the Induction of Lymphoid Tumors in C57BL Mice[1,2,3]

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Few reports on the effect of hypophysectomy on tumorigenesis have appeared in the literature. Since the pituitary is essential for growth in general, it has been suggested that the hormones produced by this gland may play an important role in neoplasia as well (*1*).

In hypophysectomized mice Korteweg and Thomas (*2*) found that the growth of transplantable and 3,4-benzpyrene-induced tumors was retarded, but concluded that the observed differences were merely of a quantitative nature. Gardner (*3*) noted that when mice bearing spontaneous mammary tumors were hypophysectomized, either during the latter half of pregnancy or postpartum, progressive growth of the tumors occurred and frequently new tumors appeared. Gardner's earlier observations have recently been confirmed and extended by Martinez and Bittner (*4*).

Moon and co-workers (*5*) observed lymphosarcomas in pulmonary and lymphatic tissues of intact rats following long-continued administration of growth hormone. Hypophysectomized rats were refractory to the tumorigenic action of growth hormone (*6*). More recently, it has been reported that hypophysectomy inhibits the development of sarcomas in rats bearing methylcholanthrene pellets (*7*), of liver tumors in rats treated with azo dyes (*8*), and of adrenal tumors in gonadectomized mice (*9*). Other investigators, using the carcinogens 9,10-dimethyl-1,2-benzanthracene (*10*) and 3,4-benzpyrene (*11*), have shown that sarcomas can be induced in hypophysectomized rats, although the rate of growth of tumors in these animals is retarded in the absence of the pituitary.

The effect of hormones on lymphoid tissue has been extensively reviewed by Dougherty (*12*), and more recently Kaplan *et al.* (*13*) have discussed the role of endocrine factors in the development of lymphoid tumors in mice.

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[1] Received for publication January 24, 1955.

[2] This investigation was supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research and the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

[3] Presented in part at the annual meeting of the American Association for Cancer Research, Inc., Atlantic City, N. J., April 10-12, 1954.

Materials and Methods

Hypophysectomies[4] were performed in immature strain C57BL mice of both sexes by the parapharyngeal method of Thomas (*14*), modified by the use of an Elliott corneal trephine with a bore of 1.5 mm. for cutting a window in the base of the skull. The mice were 30 to 40 days of age and weighed 13 to 20 gm. at the time of hypophysectomy; the experiments were started 28 days later. All hypophysectomized and unoperated animals were maintained in a warm room at 80±2° F. throughout the experimental period on a diet of bread soaked in Brewer's yeast-enriched milk,[5] lettuce, carrots, and Purina laboratory chow. The hypophysectomized animals were given 0.9-percent sodium chloride and 5-percent glucose solution as drinking fluid, while the intact mice received tap water. Animals were carefully randomized among experimental and control groups with the sexes equally distributed. X-ray treatment was given in 4 whole-body exposures of 119 r each at 8-day intervals.[6] The day of the first X-ray treatment has been designated as day 1, and day 25 is the day of the last exposure.

Four experiments were undertaken in the following order:

Experiment 1.—The weight response of lymphoid tissues following systemic irradiation in hypophysectomized and intact mice.

Experiment 2.—The effect of shielding of the thigh during X irradiation on lymphoid tissue regeneration in hypophysectomized mice.

Experiment 3.—The effect of hypophysectomy on the induction of radiation-induced lymphoid tumors in mice.

Experiment 4.—The comparative effect of thigh shielding on lymphoid tumor incidence in hypophysectomized and intact mice.

In the first two experiments animals were sacrificed with ether at designated intervals after irradiation, and their lymphoid tissues (4 pooled superficial lymph nodes, thymus, and spleen) were dissected free of extraneous tissue and weighed on a Roller-Smith torsion balance. In the tumor-induction experiments, animals that became moribund or died were autopsied routinely, and representative tissues were taken whenever the presence or absence of lymphoid tumors could not be established by gross inspection. These tissues were fixed in Bouin's fluid and processed for histologic study.

Completeness of hypophysectomy was checked against several criteria. In experiments 1 and 2, male animals having testicular weights greater than 38.0 mg. and seminal vesicle weights greater than 6.0 mg., and females having uterine weights greater than 8.0 mg. were rejected from the experimental results. In experiments 3 and 4, serial body-weight determinations were made at 28-day intervals and animals showing steady increase in body weight were eliminated. In addition to the body-

[4] The authors are grateful to Dr. W. U. Gardner for his helpful suggestions concerning the technique of performing hypophysectomies on mice.

[5] The proportions were as follows: 2 quarts homogenized milk, 2.5 standard large loaves white bread, 50 gm. Brewer's yeast.

[6] Physical factors were: 120 KVP, 9 Ma., 0.25 mm. Cu and 1.0 mm. Al added filter, 30 cm. mouse-target distance, 32 r/min.

TABLE 1.—*Mean absolute and rela*

Expt. day †	FEMALES					
	Hypophysectomized		Hypophysectomized + X ray*		Intact	
	Absolute weight‡	Relative weight§	Absolute weight	Relative weight	Absolute weight	
26	34.6± 5.7 (6)‖	214.7± 26.3	12.1± 2.8 (3)	90.3±21.5	54.3± 7.5 (3)	245
27	37.9± 0.9 (4)	264.3± 29.8	11.7± 1.3 (4)	83.0±10.2	61.4± 5.1 (5)	281
29	36.3± 8.7 (5)	241.0± 18.6	12.4± 2.1 (4)	100.0± 6.3	54.0± 3.0 (4)	243
33	34.4± 4.1 (4)	228.0± 19.6	11.9± 1.4 (4)	82.3±15.7	53.7± 5.9 (4)	219
41	33.8± 9.5 (5)	229.0± 94.6	7.5± 3.3 (5)	51.3±16.8	51.4± 9.7 (5)	233
57	28.6± 2.6 (5)	210.8± 36.5	8.8± 5.3 (4)	61.5±40.0	38.4± 4.1 (5)	163
89	30.4± 4.9 (3)	193.3± 42.5	17.8± 2.5 (4)	114.3±17.8	49.7± 7.2 (5)	189
26	10.7± 2.8 (6)‖	66.7± 10.4	4.3± 1.7 (3)	37.7± 2.6	15.6± 0.7 (3)	70
27	11.4± 4.0 (4)	78.3± 20.1	6.0± 1.3 (4)	41.0± 5.4	15.8± 1.3 (5)	75
29	12.6± 3.9 (5)	90.0± 19.5	3.7± 0.8 (4)	29.8± 4.9	18.7± 5.1 (4)	83
33	12.6± 5.8 (4)	84.3± 34.7	3.6± 0.9 (4)	24.8± 7.4	14.1± 0.9 (4)	58
41	11.7± 5.6 (5)	69.5± 53.7	3.3± 1.4 (5)	24.5± 8.2	14.6± 2.4 (5)	70
57	14.2± 3.5 (5)	111.0± 43.6	5.4± 5.3 (4)	39.5±39.1	11.8± 2.6 (5)	49
89	9.8± 0.9 (3)	62.7± 9.6	7.8± 2.4 (4)	49.3±15.2	12.3± 1.0 (5)	48
26	40.6± 4.3 (6)	267.0± 15.2	19.7± 4.1 (3)	161.7± 0.7	98.4±12.3 (3)	443
27	49.0± 3.5 (4)	341.6± 47.3	24.9± 5.5 (4)	176.8±40.3	96.0±12.3 (5)	453
29	45.8±10.3 (5)	317.5± 38.6	24.1± 4.5 (4)	196.5±42.4	105.8±19.1 (4)	473
33	45.8± 4.1 (4)	305.6± 33.4	32.6± 8.0 (4)	226.8±67.8	103.3±11.3 (4)	423
41	38.2± 2.1 (5)	266.3± 35.0	30.2±12.1 (5)	212.3±59.1	99.1± 4.2 (5)	463
57	41.0±10.0 (5)	310.8±115.6	18.0± 1.3 (4)	126.0± 9.8	84.8± 5.7 (5)	373
89	37.5± 3.7 (3)	236.0± 22.5	27.5±11.9 (4)	157.0±35.6	96.8±10.3 (5)	363

*X-ray dose: 119 r × 4 q. 8 days.

†Day 25 is the day of the last X-ray exposure.

‡Absolute weight in mg.: figure given below is $\tilde{\sigma}$, a range estimate of the standard deviation, not of the standard error of the tions; k_n is a factor given in table 16-4 of Dixon and Massey, p. 239 (*11*).

ute and relative thymus, lymph-node, and spleen weights of irradiated and untreated hypophysectomized or in

Thymus

Intact		Intact+X ray*		Hypophysectomized		Hypophyse
te t	Relative weight	Absolute weight	Relative weight	Absolute weight	Relative weight	Absolute weight
7. 5	245. 0±34. 7	21. 2± 3. 3 (5)	105. 7±12. 9	33. 8± 0. 2 (3)	264. 3± 3. 9	12. 3± 3. (5)
5. 1	281. 3±27. 5	16. 6± 2. 7 (5)	82. 3±11. 6	32. 6± 4. 2 (4)	235. 8± 19. 5	8. 9± 5. (5)
3. 0	243. 0±17. 3	22. 7± 3. 5 (5)	120. 8±17. 8	38. 2± 6. 4 (5)	268. 8± 52. 8	8. 9± 2. (6)
5. 9	219. 5±23. 4	26. 3± 8. 2 (4)	126. 5±35. 9	37. 0± 3. 4 (4)	213. 5± 23. 3	13. 7± 1. (4)
9. 7	233. 5±37. 5	41. 9± 9. 1 (5)	198. 3±53. 7	35. 1± 7. 6 (4)	230. 0± 30. 1	18. 7±14. (4)
4. 1	163. 8±18. 8	48. 9±13. 8 (5)	216. 3±51. 8	34. 7± 9. 8 (4)	243. 8± 21. 1	7. 0± 1. (5)
7. 2	189. 7± 6. 2	47. 2±12. 1 (4)	183. 7±46. 7	31. 2± 4. 9 (4)	170. 0± 19. 3	15. 6± 3. (4)

Lymph Node

0. 7	70. 3± 1. 6	6. 2± 1. 5 (5)	26. 3± 5. 3	12. 8± 2. 6 (3)	100. 3± 18. 5	4. 8± 1. (5)
1. 3	75. 5±10. 8	6. 24± . 7 (5)	30. 0±11. 3	12. 5± 5. 0 (4)	90. 5± 34. 2	2. 8± 2. (5)
5. 1	83. 8±16. 1	7. 0± 1. 5 (5)	37. 5± 7. 3	12. 5± 2. 7 (5)	91. 2± 20. 1	3. 4± 0. (6)
0. 9	58. 5± 8. 7	7. 5± 3. 4 (4)	33. 5±13. 4	10. 1± 2. 0 (4)	55. 5± 9. 3	2. 9± 0. (4)
2. 4	70. 0±15. 3	7. 1± 0. 8 (5)	35. 0± 5. 4	9. 3± 1. 9 (4)	62. 0± 15. 3	6. 9± 3. (4)
2. 6	49. 2±12. 9	12. 6± 8. 3 (5)	62. 8±40. 3	8. 9± 1. 7 (4)	63. 5± 9. 1	5. 2± 2. (5)
1. 0	48. 3± 8. 0	14. 2± 3. 9 (4)	51. 0±18. 2	13. 4± 1. 6 (4)	72. 0± 11. 9	6. 3± 1. (4)

Spleen

12. 3	443. 0±47. 8	47. 3± 5. 6 (5)	240. 0±21. 5	36. 6± 1. 9 (3)	286. 0± 8. 0	18. 9± 2. (5)
12. 3	452. 0± 8. 5	43. 1± 3. 7 (5)	213. 8±19. 2	42. 7±11. 1 (4)	303. 0± 21. 1	21. 7± 9. (5)
19. 1	478. 8±98. 5	62. 2± 6. 5 (5)	326. 3±42. 8	41. 8± 5. 7 (5)	295. 5± 45. 0	25. 2±12. (6)
11. 3	427. 5±51. 6	59. 8± 1. 3 (4)	287. 5±14. 5	54. 8±17. 0 (4)	318. 8±103. 3	29. 8±11. (4)
4. 2	460. 0±31. 9	71. 7±11. 1 (5)	339. 0±41. 2	41. 9± 8. 7 (4)	274. 0± 28. 9	31. 5± 7. (4)
5. 7	372. 5±11. 7	70. 1±12. 6 (5)	318. 8±57. 4	44. 0± 4. 9 (4)	325. 3± 85. 2	20. 1± 1. (5)
10. 3	365. 7±54. 0	86. 2±17. 3 (4)	319. 7±91. 4	40. 2± 6. 9 (4)	220. 7± 26. 9	29. 0± 5. (4)

‡Relative weight in mg. per 100 gm. terminal body weight; fig
‖Indicates number of mice in group.

dard error of the mean. $\delta = k_n r_n$ where r_n is the range of n observa-

nd relative thymus, lymph-node, and spleen weights of irradiated and untreated hypophysectomized or intact strain C57BL mice

Thymus

	MALES									
Intact		Intact+X ray*		Hypophysectomized		Hypophysectomized+X ray*		Intact		Intact+X ray*	
	Relative weight	Absolute weight	Relative weight	Absolute weight	Relative weight	Absolute weight	Relative weight	Absolute weight	Relative weight	Absolute weight	Relative weight
	245. 0±34. 7	21. 2± 3. 3 (5)	105. 7±12. 9	33. 8± 0. 2 (3)	264. 3± 3. 9	12. 3± 3. 3 (5)	86. 0±25. 2	47. 0± 6. 1 (5)	160. 3±17. 2	16. 1± 4. 3 (5)	70. 3±11. 8
	281. 3±27. 5	16. 6± 2. 7 (5)	82. 3±11. 6	32. 6± 4. 2 (4)	235. 8± 19. 5	8. 9± 5. 3 (5)	59. 8±33. 2	42. 5± 3. 0 (4)	166. 5±14. 1	10. 2± 1. 9 (5)	37. 5± 5. 9
	243. 0±17. 3	22. 7± 3. 5 (5)	120. 8±17. 8	38. 2± 6. 4 (5)	268. 8± 52. 8	8. 9± 2. 7 (6)	70. 5± 8. 6	39. 8± 6. 6 (4)	145. 0±22. 6	14. 4± 2. 8 (6)	56. 0± 9. 6
	219. 5±23. 4	26. 3± 8. 2 (4)	126. 5±35. 9	37. 0± 3. 4 (4)	213. 5± 23. 3	13. 7± 1. 8 (4)	93. 8±13. 7	44. 8± 5. 2 (5)	175. 5±17. 2	21. 7± 3. 0 (4)	82. 5±12. 4
	233. 5±37. 5	41. 9± 9. 1 (5)	198. 3±53. 7	35. 1± 7. 6 (4)	230. 0± 30. 1	18. 7±14. 8 (4)	125. 5±84. 2	35. 0± 1. 0 (4)	159. 0± 8. 1	21. 0± 3. 8 (4)	83. 0±12. 9
	163. 8±18. 8	48. 9±13. 8 (5)	216. 3±51. 8	34. 7± 9. 8 (4)	243. 8± 21. 1	7. 0± 1. 0 (5)	51. 0±11. 5	35. 0± 1. 0 (4)	130. 8± 4. 7	35. 9± 8. 4 (5)	133. 5±25. 6
	189. 7± 6. 2	47. 2±12. 1 (4)	183. 7±46. 7	31. 2± 4. 9 (4)	170. 0± 19. 3	15. 6± 3. 5 (4)	112. 0±17. 0	35. 5± 2. 7 (5)	124. 3± 5. 9	41. 0± 4. 6 (5)	132. 3±10. 1

Lymph Node

70. 3± 1. 6	6. 2± 1. 5 (5)	26. 3± 5. 3	12. 8± 2. 6 (3)	100. 3± 18. 5	4. 8± 1. 5 (5)	31. 7±11. 1	12. 8± 1. 4 (5)	45. 3± 3. 6	6. 6± 2. 2 (5)	20. 3± 9. 2
75. 5±10. 8	6. 24± . 7 (5)	30. 0±11. 3	12. 5± 5. 0 (4)	90. 5± 34. 2	2. 8± 2. 1 (5)	16. 0±12. 0	17. 9± 5. 8 (4)	70. 5±23. 1	6. 7± 1. 0 (5)	26. 5± 5. 3
83. 8±16. 1	7. 0± 1. 5 (5)	37. 5± 7. 3	12. 5± 2. 7 (5)	91. 2± 20. 1	3. 4± 0. 6 (6)	29. 0± 6. 7	18. 1± 6. 3 (4)	68. 8±23. 1	5. 6± 1. 9 (6)	25. 5± 7. 1
58. 5± 8. 7	7. 5± 3. 4 (4)	33. 5±13. 4	10. 1± 2. 0 (4)	55. 5± 9. 3	2. 9± 0. 4 (4)	19. 8± 3. 9	11. 6± 3. 5 (5)	44. 0±11. 8	5. 4± 1. 2 (4)	20. 2± 3. 7
70. 0±15. 3	7. 1± 0. 8 (5)	35. 0± 5. 4	9. 3± 1. 9 (4)	62. 0± 15. 3	6. 9± 3. 2 (4)	48. 0±18. 9	15. 8± 5. 0 (4)	57. 5±17. 2	7. 2± 1. 9 (4)	28. 8± 2. 2
49. 2±12. 9	12. 6± 8. 3 (5)	62. 8±40. 3	8. 9± 1. 7 (4)	63. 5± 9. 1	5. 2± 2. 2 (5)	34. 2± 9. 9	16. 3± 3. 0 (4)	60. 8± 9. 4	6. 9± 1. 5 (5)	25. 8± 4. 9
48. 3± 8. 0	14. 2± 3. 9 (4)	51. 0±18. 2	13. 4± 1. 6 (4)	72. 0± 11. 9	6. 3± 1. 9 (4)	46. 3± 9. 1	13. 1± 1. 2 (5)	45. 7± 5. 6	13. 5± 3. 4 (5)	46. 3± 8. 2

Spleen

443. 0±47. 8	47. 3± 5. 6 (5)	240. 0±21. 5	36. 6± 1. 9 (3)	286. 0± 8. 0	18. 9± 2. 0 (5)	148. 7±14. 5	80. 4± 9. 2 (5)	278. 3±15. 1	36. 0± 0. 7 (5)	149. 7±13. 8
452. 0± 8. 5	43. 1± 3. 7 (5)	213. 8±19. 2	42. 7±11. 1 (4)	303. 0± 21. 1	21. 7± 9. 6 (5)	155. 3±45. 2	72. 1± 5. 3 (4)	263. 5±35. 9	40. 7± 3. 5 (5)	153. 3±18. 9
478. 8±98. 5	62. 2± 6. 5 (5)	326. 3±42. 8	41. 8± 5. 7 (5)	295. 5± 45. 0	25. 2±12. 0 (6)	213. 5±57. 4	70. 9±10. 5 (4)	270. 8±25. 8	54. 3± 5. 1 (6)	223. 8±21. 9
427. 5±51. 6	59. 8± 1. 3 (4)	287. 5±14. 5	54. 8±17. 0 (4)	318. 8±103. 3	29. 8±11. 3 (4)	197. 3±37. 9	74. 3± 4. 3 (5)	291. 8±11. 8	54. 3± 4. 1 (4)	207. 0±22. 2
460. 0±31. 9	71. 7±11. 1 (5)	339. 0±41. 2	41. 9± 8. 7 (4)	274. 0± 28. 9	31. 5± 7. 5 (4)	223. 8±22. 7	83. 5±10. 7 (4)	306. 0±28. 7	59. 0± 2. 6 (4)	233. 8± 6. 5
372. 5±11. 7	70. 1±12. 6 (5)	318. 8±57. 4	44. 0± 4. 9 (4)	325. 3± 85. 2	20. 1± 1. 5 (5)	140. 5±13. 1	70. 3± 4. 4 (4)	262. 3±18. 9	58. 8± 6. 5 (5)	217. 3±13. 6
365. 7±54. 0	86. 2±17. 3 (4)	319. 7±91. 4	40. 2± 6. 9 (4)	220. 7± 26. 9	29. 0± 5. 0 (4)	203. 0±25. 9	67. 8± 6. 6 (5)	237. 3±21. 2	62. 8± 7. 9 (5)	218. 0±17. 0

‡Relative weight in mg. per 100 gm. terminal body weight; figure given below is *s*, standard deviation of observations, not standard error of mean. $s=\sqrt{\frac{\Sigma(X_i-\overline{X})^2}{N-1}}$

‖Indicates number of mice in group.

ror of the mean. $\dot{s}=k_n r_n$ where r_n is the range of n observa-

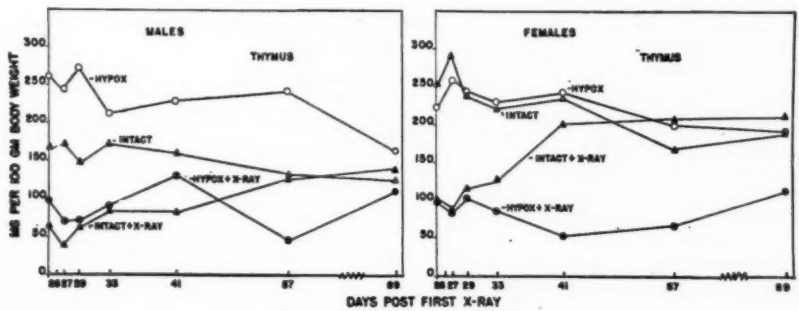
weight determinations, histologic studies were made of lymphoid organs, gonads, uteri, seminal vesicles, adrenals, and kidneys from all hypophysectomized animals. Animals that did not exhibit a typical histologic picture of severe atrophy of target organs were excluded. In a few cases in which body weight was suspiciously high and the organs appeared atrophic, the sella turcica and its surrounding contents were examined histologically and the animals were included or rejected on the basis of the sections. Approximately 15 percent of the hypophysectomies performed in these experiments were judged incomplete according to the criteria listed.

Results[7]

Experiment 1.—The absolute and relative weights of the superficial lymph nodes, thymus, and spleen are presented in table 1, and the relative weights are shown graphically in text-figures 1, 2, and 3. One intact and one hypophysectomized group of mice were systemically irradiated while another set of each served as controls. All were sacrificed from 1 to 64 days after the last X-ray exposure. There were 3 to 6 animals in each of the 4 groups at every sacrifice point. It can be seen that there is a persistence of significant relative thymic hypertrophy (text-fig. 1) in the hypophysectomized males, whereas in the females relative thymic weights are maintained at approximately control levels.

The radiosensitivity of the thymus, as measured by relative weight reduction at intervals after the last X-ray exposure, appears to be increased by hypophysectomy. Thymic recovery is significantly inhibited in the female hypophysectomized irradiated group as compared with the intact irradiated females, whereas in the hypophysectomized males there appears to be no augmented interference with regeneration after irradiation.

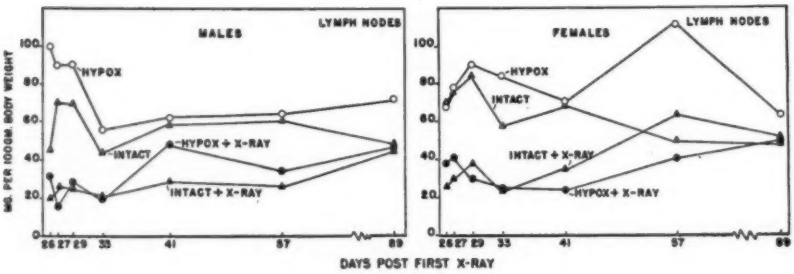
Hypophysectomy also increases relative lymph-node weights (text-fig. 2), but the initial weight loss associated with irradiation injury appears to be unaffected by hypophysectomy. Regeneration of lymph-node tissue



TEXT-FIGURE 1.—Thymus weights of irradiated and untreated hypophysectomized and intact strain C57BL mice sacrificed at serial intervals after irradiation.

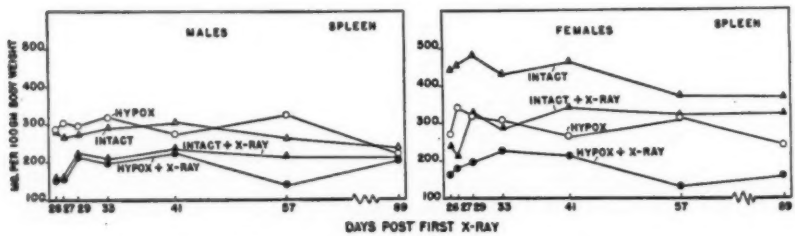
[7] Grateful acknowledgment is made to Dr. Lincoln Moses, Assistant Professor of Statistics, Stanford University, for performing the statistical analyses of the following experiments.

is significantly inhibited in the female hypophysectomized irradiated group but not in the males.



TEXT-FIGURE 2.—Superficial lymph-node weights of the same groups as in text-figure 1. Pooled weights of 2 axillary and 2 inguinal nodes.

Relative splenic weight (text-fig. 3) is significantly reduced in the hypophysectomized female controls but not in the males. Weight loss due to radiation injury is more pronounced among females of both hypophysectomized and intact groups than in the males of the corresponding groups. Regeneration of splenic tissue after irradition is also inhibited in the females.



TEXT-FIGURE 3.—Spleen weights of the same groups as in text-figure 1.

The relative weight responses of other organs (adrenals, kidneys, ovaries, uterus, testes, and seminal vesicles) following systemic irradiation in hypophysectomized and intact strain C57BL mice have been reported elsewhere (*13*).

Experiment 2.—This experiment was carried out to determine whether shielding the thigh during irradiation could accelerate lymphoid-tissue recovery in hypophysectomized mice, as it has previously been observed to do in intact mice.

The results of this experiment may be seen in table 2. All the mice were hypophysectomized. One group was exposed to total-body irradiation, while a second group was irradiated, the thigh being shielded by a strip of lead 1 cm. wide and 3 mm. in thickness. A third group served as untreated age controls. All animals were sacrificed 57 days after the first X-ray exposure. The lymphoid tissues of the thigh-shielded group

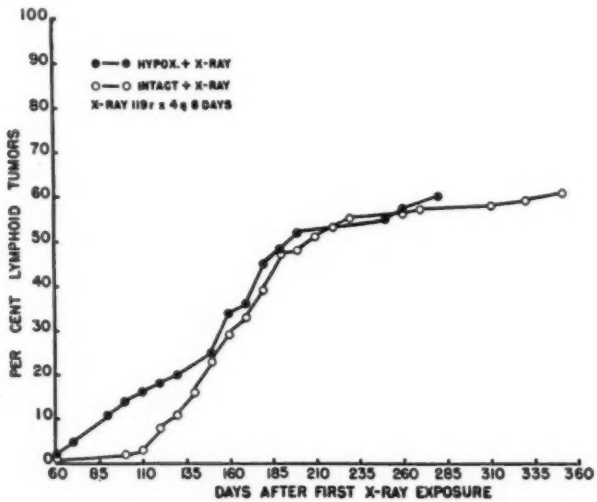
TABLE 2.—*Effect of thigh shielding during X irradiation on lymphoid-tissue recovery in hypophysectomized strain C57BL mice*

Group *	Females					Males				
	No. mice	Mean body wt. gm.	Nodes	Thymus	Spleen	No. mice	Mean body wt. gm.	Nodes	Thymus	Spleen
Hypox. control	7	16. 2	58†	199	246	3	15. 0	87	215	253
Hypox. +X ray‡ (thigh-shielded)	8	14. 6	74	251	237	7	16. 5	77	225	210
Hypox. +X ray‡ (whole-body)	6	14. 8	43	101	162	6	14. 6	45	76	166

*All hypophysectomies performed at 33 ± 3 days of age.
†Mean organ weights in mg./100 gm. body weight at 57 days after first X-ray exposure.
‡X-ray dose 119 r × 4 q. 8 days, 30 ± 3 days after hypophysectomy.

had recovered to control weight levels, while those of the total-body irradiated group were still markedly involuted at this time (table 2).

Experiment 3.—One hypophysectomized and one intact group of mice were given total-body irradiation, while a third group of hypophysectomized mice was untreated. All were observed for the development of lymphoid tumors. The results are presented in table 3. Approximately 60 percent of the animals in both the hypophysectomized irradiated and intact irradiated groups developed lymphoid tumors. The cumulative incidence curves are shown in text-figure 4. The mean latent period (160 days post first X-ray exposure) for the hypophysectomized irradiated groups was slightly less than for the intact irradiated group (180 days post first X-ray treatment). While there appears to be a slightly earlier appearance of lymphoid tumors in the hypophysectomized irradiated than in the intact irradiated group, this has not been shown to be statistically significant.



TEXT-FIGURE 4.—Lymphoid-tumor development in irradiated hypophysectomized and intact strain C57BL mice.

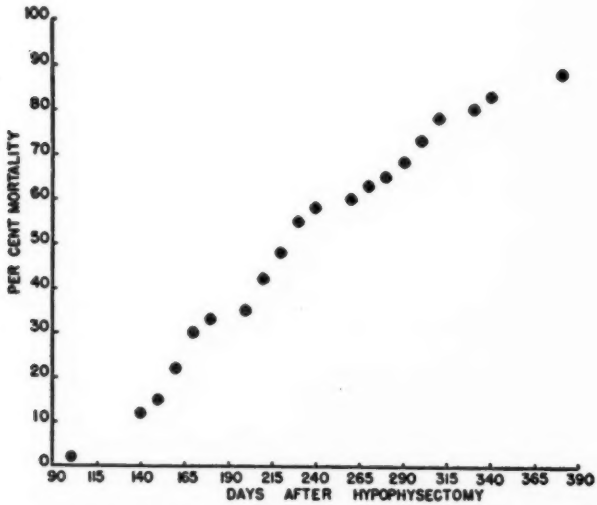
Survival of hypophysectomized untreated age controls was checked and the percent mortality over a period of approximately 1 year is shown in text-figure 5. Fifty percent of the animals for this group survived over 215 days after hypophysectomy, with only 12-percent survival over 360 days. No typical lymphoid tumors were seen in this group, although 11 out of 40 animals exhibited a peculiar hyperplastic change in their lymph nodes several months following hypophysectomy, with what appeared to be masses of reticulum cells largely replacing some of the nodes, though

not completely obliterating their architecture. The resemblance to a benign neoplastic change was suggestive, but more detailed histologic study will be necessary before a definite diagnosis can be made.[8]

TABLE 3.—*Effect of hypophysectomy on incidence of radiation-induced lymphomas in strain C57BL mice*

Group	Net number of mice	Mice without lymphomas	Lymphomas		Mean latent period*
			(*number*)	(*percent*)	
Hypox. control †	40	40	0	0	191 ‡
Hypox. + X ray †, §, ‖	43	17	26	*60. 4*	160. 7
Intact + X ray§	88	12	53	*60. 2*	180. 7

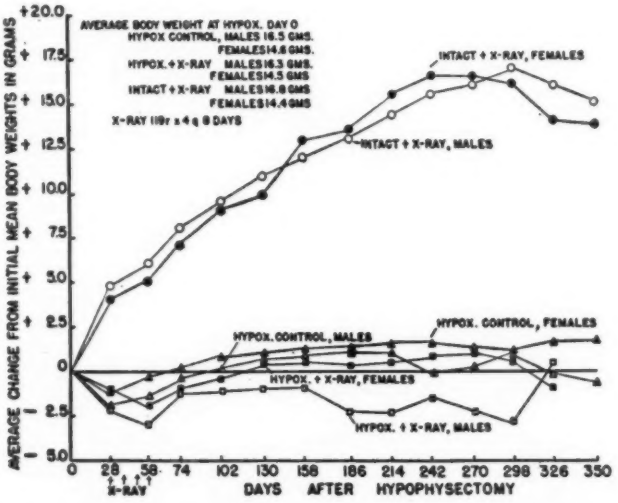
*Days after first X-ray exposure.
†All hypophysectomies performed at 33 ± 3 days of age.
‡Mean survival period.
§X-ray dose 119 r × 4 q. 8 days.
‖X-ray treatment started at 30 ± 3 days after hypophysectomy.



TEXT-FIGURE 5.—Cumulative mortality of untreated hypophysectomized strain C57BL mice. Hypophysectomy at 30 to 40 days of age.

[8] A very small sample of this material has been examined histologically by Dr. William H. Carnes, Associate Professor of Pathology, Stanford University School of Medicine, and Dr. Thelma B. Dunn, of the National Cancer Institute, who tentatively suggested that the lymph nodes might be involved by a foreign body reaction or by the type C reticulum-cell neoplasm, similar to that described by Dr. Dunn in her review (J. Nat. Cancer Inst. 14: 1281–1433, 1954).

Mean body-weight changes of animals from all groups were recorded at 28-day intervals over a period of 350 days. The mean body-weight change has been expressed as the average change from the initial mean body weight, *i.e.*, weight at the time the animals were hypophysectomized (text-fig. 6). Both the irradiated and control hypophysectomized groups exhibited an initial weight loss at 28 days and then remained fairly stationary over the next several months, while the irradiated intact animals continued to gain weight up to about 10 months of age. The weight data provided a good indication that the animals had been completely hypophysectomized.



TEXT-FIGURE 6.—Body weights of irradiated, untreated hypophysectomized, and intact C57BL mice at serial intervals after hypophysectomy at 30 to 40 days of age.

Experiment 4.—Thirty-eight hypophysectomized and 53 intact animals were exposed to total-body irradiation, the thigh being shielded by lead, and observed for the development of lymphoid tumors. During the observation period of 13 months only 2 mice from the hypophysectomized and 3 mice from the intact group developed lymphomas (table 4). This demonstrates clearly that leukemogenesis is inhibited by thigh shielding just as effectively in hypophysectomized as in intact mice.

For those interested in the comparative organ-weight responses in completely and incompletely hypophysectomized animals, the data from a representative sample of mice of each category have been included (table 5).

Discussion

Experiments 1 and 2 indicate that the pituitary is not necessary for the maintenance or regeneration of lymphoid tissues. Of particular interest is the fact that relative lymphoid-tissue weights of hypophysectomized

TABLE 4.—*Effect of thigh shielding on incidence of radiation-induced lymphomas in hypophysectomized and intact strain C57BL mice*

Group	Net number of mice	Mice without lymphomas	Mice with lymphomas		Latent periods*
			(*number*)	(*percent*)	
Hypox.† + X ray ‡ (thigh-shielded).	38	36	2	5.3	231 257
Intact§ + X ray ‡ (thigh-shielded)..	53	50	3	5.7	210 338 378

*Days after first X-ray exposure.
†Hypophysectomies performed at 36 ± 3 days of age; X-ray treatment started 30 ± 3 days after hypophysectomy.
‡X-ray dose 119 r × 4 q. 8 days.
§Mice 66 ± 3 days of age (same as hypox. group) at start of X-ray treatment.

strain C57BL mice are greater than or equal to those of intact animals of the same age, despite the fact that the hypophysectomized animal loses from 10 to 20 percent of its body weight during the first month, subsequently fails to gain much beyond its initial weight, and all its other organs exhibit marked atrophy (*13*). In the case of the thymus and lymph nodes, the relative weight increase in males was statistically significant. The spleens of hypophysectomized males showed a relative weight almost the same as that of intact males of the same age, while in hypophysectomized females relative splenic weight was reduced. A number of published studies have been in disagreement as to whether the thymi of hypophysectomized rats are smaller or relatively larger than those of intact animals of comparable age (*12*). Our results in strain C57BL mice lend support to those studies in which relative thymic and lymph node hypertrophy was noted following removal of the pituitary.

It has been shown that adrenalectomy, gonadectomy, or both adrenalectomy and gonadectomy cause a distinct increase in lymphoid-tissue weight in rats and mice (*13*, *15*, *16*). Thyroidectomy in rats has been reported to produce a slight loss in lymphoid-tissue weight (*17*), whereas thyroxin has a trophic effect on lymphoid tissues, especially in adrenalectomized animals (*18*, *19*). Either growth hormone or thyrotrophin administered to intact, hypophysectomized, or adrenalectomized, gonadectomized mice may cause an increase in relative weights of lymphoid tissues.[9] The pituitary, therefore, appears to exert an indirect influence by means of its endocrine-trophic factors in the regulation of lymphoid tissue growth in normal animals.

Dougherty, in an excellent review (*12*), emphasizes the importance of taking into consideration the methods of analysis of data employed by various investigators. For example, when lymphoid tissues of hypophysectomized animals are expressed on an absolute weight basis, one fails to see any increase in lymphoid-tissue weight (table 1); however, if the results are expressed in mg. per 100 gm. body weight (relative weight),

[9] C. S. Nagareda and H. S. Kaplan—unpublished observations.

TABLE 5.—*Absolute body and organ weights from sample hypophysectomized and incompletely hypophysectomized strain C57BL mice**

Mice (*number*)	Body wt. at hypox.† (*gm.*)	Body wt. 28 days after hypox.	Body wt. 53 days after hypox.	Body wt. at sacrifice‡	Nodes (*mg.*)	Thymus (*mg.*)	Spleen (*mg.*)	Kidney (left) (*mg.*)	Adrenals (*mg.*)	Testes, ovaries (*mg.*)	Seminal vesicle, uterus (*mg.*)
Males, completely hypophysectomized											
117	15. 8	12. 4	12. 8	12. 2	13. 4	31. 1	34. 4	44. 0	2. 0	5. 0	2. 0
118	20. 0	17. 7	17. 2	17. 3	9. 0	36. 5	57. 2	61. 2	2. 0	16. 5	5. 0
230	16. 6	13. 2	12. 9	14. 2	11. 8	28. 0	33. 4	46. 6	1. 4	12. 5	2. 1
275	15. 2	12. 6	13. 3	13. 6	11. 8	46. 7	34. 4	61. 5	1. 5	19. 3	2. 9
280	15. 4	13. 6	14. 0	13. 0	11. 8	35. 2	47. 6	57. 1	2. 2	20. 3	2. 4
370	13. 2	12. 0	12. 5	12. 6	15. 0	34. 0	35. 0	48. 8	2. 2	11. 0	1. 5
Males, incompletely hypophysectomized											
116	19. 6	*20. 5*§	*23. 9*	*23. 6*	17. 5	51. 5	*89. 9*	*137. 0*	*2. 9*	*165. 1*	*26. 0*
157	18. 8	15. 4	16. 8	17. 1	9. 2	34. 9	52. 6	66. 3	*3. 0*	*115. 9*	*92. 8*
223	16. 9	*21. 2*	*25. 1*	*36. 3*	10. 4	42. 0	*77. 4*	*136. 8*	2. 4	*195. 4*	*293. 1*
276	18. 0	16. 9	18. 2	17. 4	11. 7	49. 4	47. 5	68. 5	2. 3	*121. 6*	*30. 2*
338	18. 5	*19. 8*	*22. 2*	*20. 9*	14. 6	37. 9	*69. 5*	*118. 2*	2. 4	*160. 8*	*181. 7*
371	18. 1	15. 4	17. 2	17. 4	12. 1	40. 3	45. 0	62. 5	1. 4	*105. 9*	*22. 4*
Females, completely hypophysectomized											
121	13. 9	13. 0	13. 9	14. 2	12. 5	38. 9	44. 4	48. 3	1. 0	0. 2	3. 3
122	13. 4	13. 0	14. 2	14. 6	7. 4	38. 2	50. 3	50. 0	2. 7	1. 9	4. 1
224	16. 8	15. 0	16. 0	17. 2	9. 6	30. 0	40. 7	57. 2	2. 5	1. 8	5. 1
225	16. 3	14. 7	15. 4	16. 2	9. 2	26. 5	34. 5	47. 5	2. 2	1. 3	4. 4
Females, incompletely hypophysectomized											
120	15. 2	*18. 3*	*20. 0*	*21. 4*	*24. 9*	*58. 7*	*83. 9*	*109. 0*	*5. 5*	*5. 5*	*47. 7*
123	15. 0	*16. 1*	*17. 9*	*18. 2*	14. 4	*59. 1*	*70. 3*	*74. 9*	*4. 5*	*3. 2*	*31. 2*
226	16. 0	15. 1	16. 7	*21. 4*	12. 1	36. 1	52. 6	*71. 9*	*3. 7*	*5. 4*	*33. 4*
227	14. 9	*20. 6*	*26. 8*	*36. 0*	10. 7	35. 3	*87. 6*	*114. 2*	*4. 9*	*8. 0*	*42. 2*

*Sample mice from experiment 1 of text.

†Hypophysectomies performed at 33 ± 3 days of age.

‡54–117 days after hypophysectomy.

§Figures in italics represent basis for judging mouse incompletely hypophysectomized.

an increase in lymphoid-tissue mass is noted. The results of this study bear out the point clearly. It seems logical to express organ weights in terms of relative weights when comparing hypophysectomized animals with intact controls, since a tremendous difference in body weight exists. It is of interest to note that the lymphoid organs appear to be the only tissues in the body which are maintained at essentially normal relative weight levels after hypophysectomy.

Hypophysectomy appears to increase the radiosensitivity of lymphoid tissues somewhat, and inhibits thymic recovery. The fact that lymphoid tissues are capable of regenerating in the absence of the pituitary was illustrated by shielding the thighs of hypophysectomized mice during systemic irradiation. It has been shown that thigh shielding during systemic irradiation (or intravenous injection of homologous bone marrow shortly after X-ray exposure) promotes the regeneration of lymphoid tissue (*20–22*), and also inhibits the development of lymphomas in intact strain C57BL mice (*21, 23, 24*). It is well established that bone-marrow activity is severely impaired in hypophysectomized animals, resulting in a severe anemia (*25–27*). It was interesting to find, therefore, that shielding the thigh of hypophysectomized mice during irradiation effectively promoted complete regeneration of lymphoid tissues. This observation indicates that activity of the factor in bone marrow which is responsible for postirradiation hematopoietic recovery in intact animals does not require the presence of the pituitary. This conclusion is also supported by the finding that thigh shielding apparently prevents the development of lymphomas just as effectively in hypophysectomized as in intact animals.

Experiment 3 demonstrates that the pituitary is not essential for the induction of lymphomas by irradiation. Since either gonadectomy or adrenalectomy may enhance the development of radiation-induced lymphoid tumors (*28, 29*), it might have been anticipated that hypophysectomy would also augment the incidence of lymphomas by virtue of its involutional effects on these endocrine glands. Were a method of statistical analysis available to take into consideration the short life span and increased mortality of the hypophysectomized animals after exposure to irradiation, it might be possible to demonstrate that hypophysectomy actually enhances tumor development. The fact that the tumors appear earlier in hypophysectomized than in intact animals (text-fig. 4) is suggestive though statistically inconclusive evidence for such an effect. Various investigators have reported that hypophysectomy effectively inhibits the tumorigenic action of certain carcinogenic agents (*7, 8*), and it has been suggested by Robertson *et al.* (*30*) and O'Neal *et al.* (*31*) that the pituitary-adrenal axis may be involved in the carcinogenic action of azo dyes. In this respect, it appears that the mechanism for the induction of sarcomas and liver tumors by carcinogens in rats differs from that of lymphoid-tumor induction by X irradiation in mice. In any case, our observations refute the possibility that the pituitary plays a universally essential role in carcinogenesis. The fact that radiation-induced lymphoid

tumors develop in the absence of the pituitary is consistent with our observation that growth, maintenance, and regeneration of lymphoid tissues are also independent of direct pituitary mediation in strain C57BL mice. It seems likely that hypophysectomy will significantly modify tumorigenesis only in those tissues toward which the pituitary exhibits distinct growth-conditioning activity.

Summary

1) It has been shown that growth, maintenance, and regeneration of lymphoid tissues (superficial lymph nodes, thymus, and spleen) in hypophysectomized strain C57BL mice are not dependent upon direct pituitary control.

2) Thymus weights of hypophysectomized male mice, expressed in mg. per 100 gm. body weight, significantly exceed those of intact controls of the same age. In the females no significant difference in relative thymic weight was seen. Relative lymph-node weights of hypophysectomized mice of both sexes were significantly higher than in the intact animals. Relative spleen weights were essentially the same in hypophysectomized and intact male mice of comparable age, whereas in the corresponding females there was a definite decrease following operation.

3) The radiosensitivity of lymphoid tissues of hypophysectomized mice given 4 whole-body exposures of 119 r each at 8-day intervals appears to be moderately increased by hypophysectomy. Lymphoid-tissue recovery following irradiation was slower in hypophysectomized than in intact mice.

4) The capacity of lymphoid tissues to regenerate in the absence of the pituitary has been demonstrated by the observation that hypophysectomized animals exposed to periodic systemic irradiation with their thighs shielded exhibit complete regeneration of their thymus glands, lymph nodes, and spleens within 57 days after the start of X-ray exposure, at which time involutional effects are still pronounced in the lymphoid tissues of unshielded animals.

5) It has been demonstrated that hypophysectomy does not inhibit the development of radiation-induced lymphoid tumors in strain C57BL mice. Approximately 60 percent of the mice in hypophysectomized and intact irradiated groups developed lymphomas, with a mean latent period of 160 and 180 days, respectively, after the first X-ray exposure.

6) Leukemogenesis was just as effectively inhibited in hypophysectomized as in intact mice by thigh shielding during irradiation.

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Virus of Avian Erythromyeloblastic Leukosis. VII. Thermal Stability of Virus Infectivity; of the Virus Particle; and of the Enzyme Dephosphorylating Adenosinetriphosphate [1,2]

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In the course of investigations on the virus of avian erythromyeloblastic leukosis (*1*), it has been desirable to learn the limits of the stability of the agent under various laboratory conditions. An earlier report (*2*) has described the stability of the virus with respect to hydrogen-ion concentration. Of parallel importance is information about the stability of viral properties in relation to time and temperature. An approach to the problem appearing to offer optimum potentialities for direct quantitative estimates and broad latitude for extrapolation and interpolation is the study of the stability of the virus exposed to various temperatures for differing periods of time. Accordingly, experiments were made to determine the effects of heat on the infectious properties of the agent, on the associated enzyme dephosphorylating adenosinetriphosphate (*3*), and on the physical integrity of the virus particles examined and counted in the electron microscope. The results of these experiments are described in the present report.

Materials and Methods

The experiments were made with the virus in plasma and in a concentrate of the agent sedimented a single time by ultracentrifugation of plasma. Prospective plasma donors were selected by the microdephosphorylation test (*4*). Blood was obtained from the donor birds by heart puncture with heparin as the anticoagulant. In order to avoid possible effects of preservative materials, including phenol, in commercial preparations of heparin, shift has been made to use of crystallin heparin (Heparin-sodium, 100 units per mg., Nutritional Biochemical Corporation, Cleveland, Ohio) in amounts of 1 to 2 mg. per ml. of blood. Heparin solution was prepared daily by dissolving the crystals in Simms' solution (*5*) in a concentration of 50 mg. per ml. The chilled blood of individual donors

[1] Received for publication February 3, 1955.

[2] This work was supported by research grants to Duke University from the American Cancer Society on recommendation of the Committee on Growth; from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by a gift from Lederle Laboratories Division, American Cyanamid Company; and by the Dorothy Beard Research Fund.

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was cleared of cells by centrifugation in the usual way, and pools were prepared from the plasmas of 2 to 9 donors for each of the various experiments. These plasma pools were then passed through celite and Selas filters (*6*). Virus was concentrated by spinning plasma in the ultracentrifuge at 20,000 × g for 45 minutes. The pellets were resuspended in small volumes of Ca-free Ringer's solution and spun in the angle centrifuge at 4,000 × g for 10 minutes. The preparation was then diluted to the original volume of plasma with Ca-free Ringer's solution at *p*H 7.2.

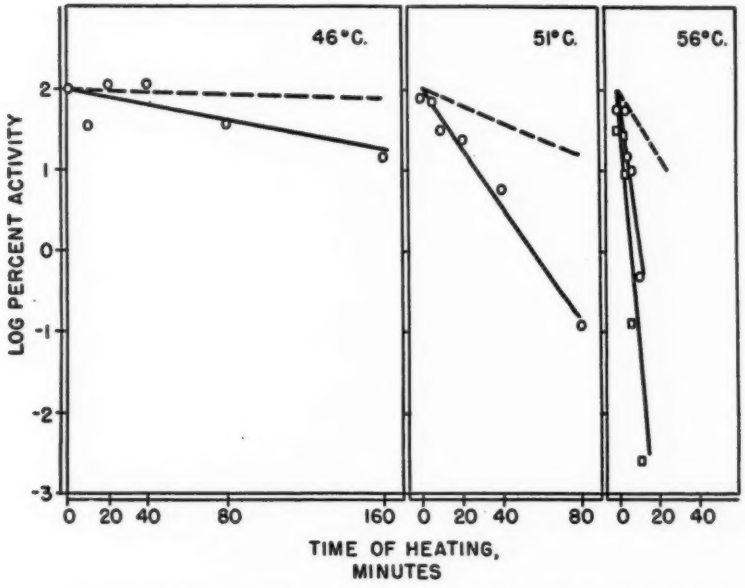
For the various studies, the virus preparation or plasmas were parceled in the appropriate number of 1.0-ml. volumes in Wassermann tubes closed with rubber stoppers. During the process of heating, the tubes were kept immersed about two thirds of their depth in the constant temperature bath. In the experiments over the longer periods, at 37° C. and below, penicillin and streptomycin were included in final concentrations of 500 units of the former and 0.1 mg. of the latter, as the sulfate, per ml. of plasma or virus preparation. A preliminary study indicated that these antibiotics were without effect on the virus.

The various specimens were analyzed, along with the suitable controls, for level of infectivity, capacity to dephosphorylate adenosinetriphosphate, and for particle content. In each instance the study was made on one of the 1.0-ml. test preparations withdrawn after appropriate intervals determined by the conditions of the various experiments. Preparations which had been heated were quickly cooled by immersion of the respective tubes in an ice bath and held there until sampled for analysis.

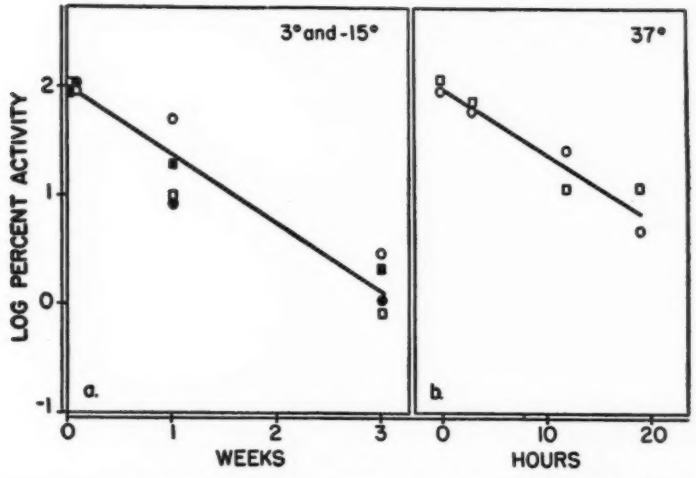
Infectivity measurements were made by the titration procedures (*7*) developed for the erythromyeloblastic leukosis virus. A portion of the 1.0-ml. test preparation was diluted to suitable volumes with BSA-Simms' solution (*8*), and 0.1-ml. volumes of the appropriate dilutions were injected intravenously into 3-day-old White Leghorn chicks [Line 15 (*9*), Regional Poultry Research Laboratory, East Lansing, Mich.]. Onset of the disease was determined by blood smears, and the level of virus infectivity was judged by the median log-latent period observed in the 30 to 40 chicks comprising each dose group. In those experiments at and above 37° C., all the samples were collected and titrated in the same weekly hatch of chicks. In the experiments at 3° C. and −15° C., titration had to be made in chicks of different weekly hatches, but all were from the same laying flock. Estimation of the ability to dephosphorylate adenosinetriphosphate was made by electrometric titration (*3*) with samples of the test mixtures. The plasma particles were examined and counted by the technique of sedimentation onto agar and electron microscopy (*6*, *10*).

Results

The scope of the experiments is shown in table 1, and the results are described graphically in text-figures 1 and 2. In these graphs, the points represent percent residual infectivity (connected by continuous lines) and enzyme activity (dash lines). These data on infectivity were derived in the following way:



TEXT-FIGURE 1.—Thermal stability, at 46° C., 51° C., and 56° C., of the infectivity (*continuous line*) and of the enzyme activity to dephosphorylate adenosinetriphosphate (*broken line*) of the virus of erythromyeloblastic leukosis in filtered whole plasma. Duplicate experiments, circles and squares, were made at 56° C.



TEXT-FIGURE 2.—Thermal stability, at 3° C. and −15° C., of the infectivity of the virus of erythromyeloblastic leukosis in whole filtered plasma. No change was observed at these temperatures in the enzyme activity of the virus to dephosphorylate adenosinetriphosphate. The *open circles* and *squares* indicate duplicate experiments at 3° C. and −15° C. and the *closed circles* and *squares* duplicate experiments at −15° C.

TABLE 1.—*Specific reaction rates of inactivation of infectivity and ATPase activity at various temperatures in plasma and BSA-Simms' solution*

Expt. No.	Medium	Particles per ml.* ($\times 10^{11}$)	Temperature (° C.)	Specific Reaction Rate, k	
				Infectivity (*Min.* $-^1$)	ATPase (*Min.* $-^1$)
117, 118, 120...	Plasma	11	−15	0. 00012†	
117, 118, 120...	Plasma	12	3	. 00012†	
123.....	Plasma	5. 7	37	. 0044	
126.....	Plasma	3. 7	37	. 0023	
131.....	Plasma	6. 3	37	. 0023	
131.....	BSA-Simms	6. 4	37	. 0021	
112.....	Plasma	.....	46	. 010	0. 00138
131.....	BSA-Simms	6. 4	46	. 018	
113.....	Plasma	6. 7	51	. 078	. 0223
98.....	Plasma	11	56	. 46	. 120
99.....	Plasma	8. 1	56	. 97	
131.....	BSA-Simms	6. 4	56	. 90	

*Concentration of virus particles in the virus preparations before treatment.

†No significant differences in stability at the two temperatures; the respective data were combined to give the rate.

Infectivity of the unheated plasma or virus was determined by inoculation of 2 dilutions into corresponding numbers of dose groups. The initial dilution injected consisted of whole plasma or virus diluted with 9 volumes of BSA-Simms' solution; the second dilution was tenfold that of the first. Measurement of residual infectivity after exposure to the conditions of the experiment was made with a single sample of each test mixture, 0.7 ml. of which was diluted with 9 volumes of BSA-Simms' solution. The volume relations with the test mixtures were thus the same as the initial dilution of unheated plasma described above. To obtain the potency ratios between the unheated and the heated preparations, there was first determined the median log-latent period of the response to the initial dilution of the unheated preparation. [The second dilution of this preparation in each experiment was used only to check the slope of the regression of median log-latent period on dose, which in all cases was not significantly different from the slope, 0.104, commonly observed (7) in these test chickens.] The median log-latent period was determined in like manner for the dose-group response to each test material. Through the plot of these points against time, the regression line was drawn by the method of least squares, and the intercept of this line with zero time was taken as representing the activity of the control unheated preparation. Employing this median log-latent period of the control preparation as representing 100-percent activity, the median log-latent periods of the responses to the various test preparations were transformed to percent activity on the basis of the standard slope, 0.104, of the regression of median log-latent period on dose as previously determined.

Inspection of the points in text-figures 1 and 2 reveals, within the limits of the method of titration, no appearance of consistent deviation of the points from linearity. The continuous lines were then drawn

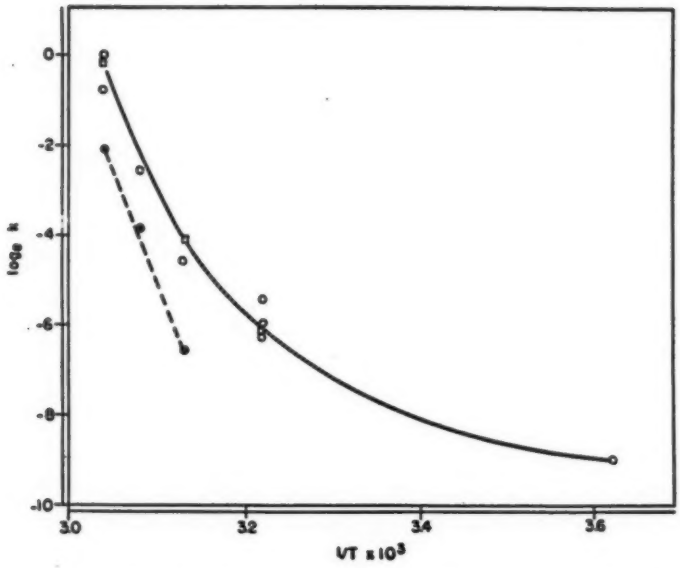
through the points by the method of least squares. The changes in enzyme activity are shown in the broken line as percent of initial dephosphorylating activity remaining after the indicated periods of heating at the respective temperatures. No significant change was observed in the particle counts under the experimental conditions and, consequently, the data were omitted from the graphs.

Rates of inactivation.—The course of inactivation of infectivity at the various temperatures is clearly shown in text-figures 1 and 2. It was evident, in all cases, that residual activity expressed as log percent of the control value, was essentially linear with the time of heating, which is characteristic of a reaction of the first order. Consequently, the slopes of these regression lines constitute the measure of the rates of inactivation at the different temperatures. Conversion of the respective values by multiplying by 2.3, gave the value, k, of the specific reaction rates shown in table 1. Table 1 shows likewise the results observed with the inactivation of the enzyme activity.

From the values cited in table 1, it can be seen that the virus of erythromyeloblastic leukosis is highly susceptible to the influence of temperature and is of low stability at any temperature within the broad range included in the present studies. No difference could be detected in the respective rates at −15° C. and 3° C.; it should be emphasized, however, that the sequence of titrations involving different groups of test birds after the various periods at these temperatures may have lessened the sensitivity of the titration procedure. As seen in text-figures 1 and 2, the estimates of potency varied more widely under these conditions than did those at higher temperatures. With increase in temperature to 37° C., the rate of inactivation was greatly accelerated, and there was no demonstrable difference between the stability of the virus in plasma and that removed from plasma and suspended in BSA-Simms' solution. Above 37° C., the rapid decline in infectivity was a matter of few minutes, whether the agent was in plasma or BSA-Simms' solution. Below 46° C., the activity of the enzyme did not diminish within the conditions of the experiments. At this temperature and above, infectivity declined at rates approximately sixfold those of the enzyme.

The data of table 1 are summarized in text-figure 3 in which the specific reaction rates are shown in comparison with the reciprocal of the absolute temperatures of the experiments (*11*). The continuous line was drawn by inspection through the points related to infectivity. It would appear that the relation was essentially linear in the region of 46° to 56° C., deviating markedly near the range of 37° C. to 46° C., but probably somewhere below 46° C. The broken line which represents the analogous graph of the behavior of enzyme activity likewise seems linear through the region of 46° C. to 56° C.

From the apparently linear relation in the restricted range of temperatures, calculations may be made (*11*) of the thermal constants for inactivation of infectivity and of enzyme activity. Within the limits of accuracy of the determinations in this region, the energies of activation of both



TEXT-FIGURE 3.—Rates of inactivation of the infectivity (*continuous line*) and of the enzyme activity to dephosphorylate adenosinetriphosphate (*broken line*) of the virus of erythromyeloblastic leukosis. The results indicated by *circles* were obtained with the virus in filtered whole plasma and those by *open squares* with virus concentrates.

attributes are essentially identical and are represented approximately by the values 88,500 cal. per mole for the destruction of infectivity and 91,000 for inactivation of the enzyme. Calculation of the entropies of activation gave values of 201 for infectivity and 207 for enzyme activity, respectively.

It is clear in text-figure 3 that, whereas the increase in the rate of inactivation at the higher temperatures is essentially a linear function of temperature, this relation does not obtain at lower temperatures. Extrapolation of the data obtained above 46° C. would indicate a far higher degree of stability than that actually observed between 46° C. and −15° C. The high rate of inactivation at 37° C. was unexpected, and several experiments were made to verify the deviation in this range. The experiments with BSA-Simms' solution were made to rule out possible effects of constituents of the plasma. With decrease in temperature, the deviations of the data became more pronounced. The true course of the curve below 46° C. is uncertain, but it seems likely that for practical purposes the relation indicated is sufficiently close to permit predictions of the stability of the virus at the various levels of temperature included.

Discussion

Certain aspects of the data of these experiments should receive special emphasis. The first of these is the range of study which includes not only the higher temperatures most often employed (*12*) for such investi-

gations but also the region of more practical significance between 37° C. and −15° C. This has been of considerable value since the findings indicate major differences in the mechanisms of inactivation at various levels of temperature. Although it was observed, as learned with other viruses (*12*), that inactivation proceeds at all temperatures as a reaction of the first order, the influence of temperature in the inactivation of the virus of erythromyeloblastic leukosis was not the same in the range below 46° C. as in that above. Consequently, the data above this level cannot be employed to predict the course of inactivation at lower temperatures. This is apparently at variance with the findings in studies of influenza A virus by Lauffer, Carnelly, and MacDonald (*13*), who regarded their data obtained above 40° C. as linear with the results obtained by Miller (*14*) at 4° C. It is important to note, however, that the rate of hypothetical inactivation of enzyme activity at 37° C. was too small for measurement after the 21-day period at this temperature. While it could not be ascertained with accuracy that the rate-temperature relation at 37° C. was linear with that at 46° C. and above, it can be judged that this relation probably did not deviate to the extent observed in the inactivation of infectivity. This suggests a difference between the mechanism of inactivation of enzyme activity and that of infectivity which implies a lack of identity of the two centers of activity in the virus particles. An analogous difference was seen in the greater stability of the enzyme under the influence of heat and, as previously found (*2*), of hydrogen-ion concentration. Still more striking is the observation (*15*) that virus infectivity can be neutralized and the particles and enzyme activity precipitated without effect on enzyme activity. Another point for emphasis is that the behavior of the enzyme of the virus of erythromyeloblastic leukosis differs greatly from that of the hemagglutinin (*16*) of the influenza A virus. With the latter agent, inactivation of the hemagglutinin appears to be a reaction of the three halves order which has been regarded as due to inhomogeneity of the property to agglutinate red cells. This evidence of inhomogeneity is lacking in the behavior of the adenosinetriphosphatase activity of the erythromyeloblastic leukosis virus.

By reference to the results of studies of other viruses (*12*), it is clear that the agent of erythromyeloblastic leukosis is very labile. It has been shown with other agents that stability may be related to the initial concentration of the virus preparation (*16*). In the present work the concentration of virus, as determined by direct particle count, table 1, varied through an approximately threefold range which was probably too narrow to have contributed to variation in the findings. It should be emphasized that in no case could inactivation be regarded as complete. With a preparation containing 10^{12} particles, inactivation of 99.9 percent of activity would have left approximately 10^{9} particles unaffected.

Summary

Studies were made on the rates of thermal inactivation of infectivity, adenosinetriphosphatase activity and the physical integrity of the particles

of the virus of avian erythromyeloblastic leukosis. The range of temperatures included those between −15° C. and 56° C. Inactivation of infectivity and enzyme activity progressed as a reaction of the first order at all temperatures. The rates of inactivation of infectivity expressed as logarithms were linear with the reciprocals of the absolute temperatures between 46° C. and 56° C., and the same relations occurred with enzyme activity. The energies of activation of the two attributes were 88,500 and 91,000 cal. per mole respectively, and the entropies of activation were 201 and 207. Below 46° C. the relation of the rate of inactivation of infectivity to temperature deviated sharply from that above this level, indicating marked lability of the virus at the lower temperatures. The findings provide evidence of lack of identity of the virus particle centers responsible respectively for infectivity and enzyme activity. No change was seen in the number or characters of the particles under the influence of the experimental conditions.

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Virus of Avian Erythromyeloblastic Leukosis. VIII. Dephosphorylation of Inosinetriphosphate[1,2]

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In work previously reported (*1*, *2*), it has been found that the blood plasma of chicks with erythromyeloblastic leukosis contains a particulate component identified as the etiologic viral agent and, in addition, the enzyme activity (*3*) to dephosphorylate adenosinetriphosphate. Further investigation has revealed an intimate association between the particles and the enzyme which appear simultaneously and proportionally with onset of the disease (*4*) and are inseparable by sedimentation or electrophoresis (*5*, *6*). The unity of particles and enzyme has been demonstrated (*7*) unequivocally by concurrent precipitation of both with specific antiviral sera from chickens hyperimmunized with the virus. In more recent work it has been observed that the enzyme, like all other adenosinetriphosphatases thus far investigated, also hydrolyzes inosinetriphosphate. The characteristics of the adenosinetriphosphatase activity have already been reported (*8*, *9*). The reactions of the enzyme with inosinetriphosphate alone and in combination with adenosinetriphosphate are described in the present paper and are compared and discussed in relation to the findings with other enzymes of analogous activity.

Materials and Methods

Plasmas of high enzyme and virus content were produced, collected, and filtered as previously described (*8*). In the present study the virus was concentrated by a single centrifugation at 20,000 × g for 45 minutes. The resulting pellets were resuspended in the minimum quantity of cold physiological saline solution and stored in the cold. The numbers of virus particles in the preparations were determined (*4*, *10*) by sedimentation onto an agar surface and by counting in electron micrographs. The activity of the enzyme in all of the experiments was determined in relation to the number of particles counted in this way.

[1] Received for publication February 3, 1955.

[2] This work was supported by a research grant to Duke University from the American Cancer Society, on recommendation of the Committee on Growth; by a grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by a grant from the Damon Runyon Cancer Memorial Fund; and by the Dorothy Beard Research Fund.

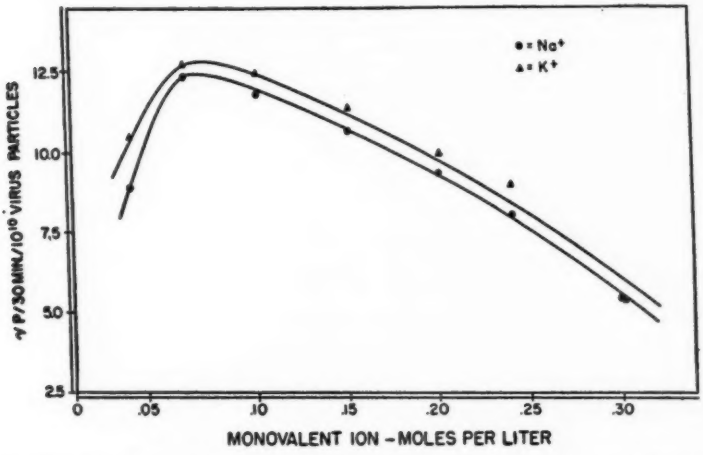
Inosinetriphosphate was obtained as the disodium salt (Sigma Chemical Co.) and adenosinetriphosphate as the dipotassium salt (Pabst Laboratories). Each nucleotide was dissolved separately in deionized water and passed over Dowex-1 (chloride cycle) contained in a 1 cm.^{2} × 4 cm. glass column fitted with a fritted glass tip. The nucleotides were then eluted free of any contaminating diphosphate, monophosphate, or phosphate by the method of Cohn and Carter (*11*). The concentrations of adenosinetriphosphate and inosinetriphosphate were estimated with the Beckman spectrophotometer at 2,600 and 2,500 Å, respectively (*12*). Each nucleotide solution was then adjusted to *p*H 7.0 with KOH and suitably diluted so that each milliliter contained 8.0 micromoles of purine base.

Studies of enzyme activity were made with preparations in a final volume of 5 ml. containing 1 ml. of 0.1 *M* buffer at the required *p*H, 3 ml. of the appropriate ion solution, 1 ml. of substrate added at zero time and 10λ of a suitably diluted virus suspension. A substrate blank eliminating the 10λ of virus was also included. No virus blank was needed since prolonged incubation of the agent alone at 30° C. did not reveal the presence of any detectable phosphate. All reaction vessels were incubated for 10 minutes at 30° C. prior to the addition of substrate. Following the addition of substrate, the reaction was allowed to proceed for such a period of time that sufficient phosphate would be produced while the reaction course was still essentially linear. The reactions were terminated by the addition of 2 ml. of 25-percent trichloroacetic acid or, in special instances, by the addition of the same volume of 2 *N* perchloric acid. The reaction mixture, together with washings, was transferred quantitatively to a 10-ml. volumetric flask and made up to volume. Filtration was not necessary, since no turbidity was produced either upon the addition of the trichloroacetic acid or following the addition of the molybdate-H_2SO_4 during the subsequent phosphate determination. Five-milliliter aliquots were used to determine phosphate by the method of Fiske and Subbarow (*13*). Rates of substrate hydrolysis were calculated by subtracting the blank value from the averages of the duplicate runs. The substrate blank was always incubated for the same period as the reaction vessel containing the enzyme.

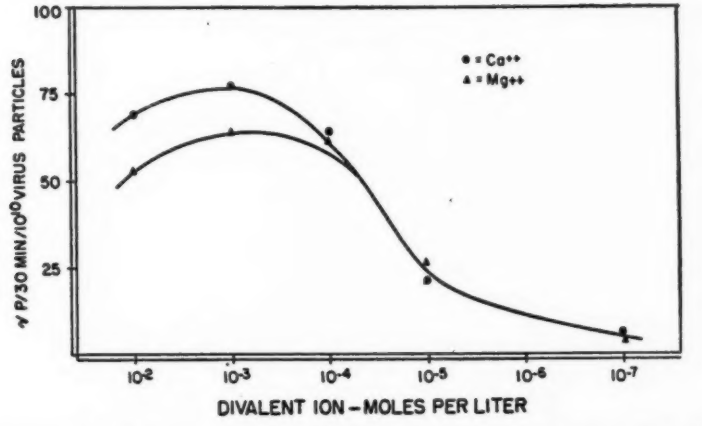
Results

Ion effects on enzyme activity.—The effects of Na^+, K^+, Ca^{++}, and Mg^{++} acting separately in the dephosphorylation of inosinetriphosphate are shown in text-figures 1 and 2. All these experiments were carried out at *p*H 7.2 in the presence of 0.02 *M* tris (trihydroxymethyl aminomethane) buffer. The results obtained in an experiment with Na^+ and another with K^+ are shown in text-figure 1. The data with the two ions were very nearly identical and showed that the effects of these monovalent ions, though definite, were relatively small. The maximal rate of the reaction occurred in the range of concentrations of 0.05 to 0.10 *M* Na^+ or K^+, and the effect of K^+ was slightly greater on both sides of the optimum than that of Na^+. The results of analogous experiments with the same virus, describing the influence of the divalent ions Ca^{++} and Mg^{++}, are

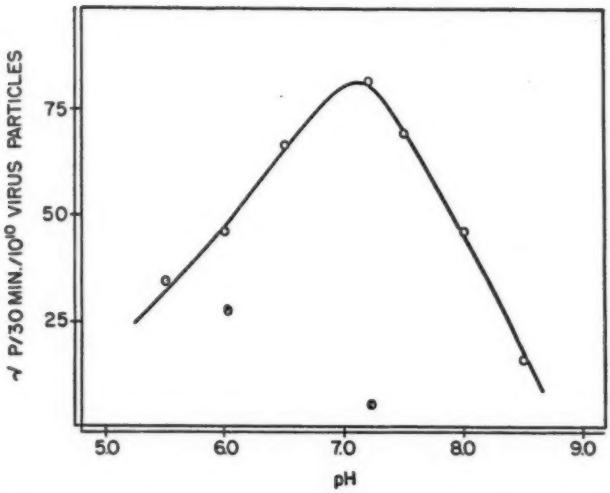
shown in text-figure 2. These experiments were carried out in the presence of 0.05 *M* Na^+ + 0.05 *M* K^+ (optimum for the monovalent ions). The activating effects of the divalent ions were considerable, the optimum occurring at about 10^{-2} to 10^{-3} *M* Ca^{++} or Mg^{++}. Ca^{++} caused a somewhat greater enhancement of activity than Mg^{++} in the region of the optimum divalent ion concentration. The activity in the presence of 0.04 *M* Ca^{++}+0.04 *M* Mg^{++}+0.05 *M* Na^++0.05 *M* K^+ was no different from that obtained with the same concentration of Ca^{++} or Mg^{++} in the presence of 0.05 *M* Na^++0.05 *M* K^+. This can be seen in text-figures 2 and 3, and the finding demonstrates the lack of any anomalous ionic



TEXT-FIGURE 1.—Influence of various concentrations of NA^+ or K^+ on the ITPase activity of the concentrated virus of erythromyeloblastic leukosis.



TEXT-FIGURE 2.—Influence of the concentration of Ca^{++} or Mg^{++} on the ITPase activity of the concentrated virus of erythromyeloblastic leukosis.



TEXT-FIGURE 3.—Influence of hydrogen-ion concentration on the ITPase activity of the concentrated virus of erythromyeloblastic leukosis in the presence of Na^+ and K^+, each at 0.05 *M* concentration, and Ca^{++} and Mg^{++}, each at 0.04 *M*. The lower single point indicates activity in the absence of the ions.

interactions. Text-figure 3 also shows a 15-fold activation at *p*H 7.2 when all 4 ions are used at optimal concentrations instead of water.

Influence of hydrogen-ion concentration.—Experiments were made to determine the effects of *p*H on the rate of the enzyme reaction in the presence of the optimal concentrations of Na^+, K^+, Ca^{++}, and Mg^{++} as indicated in text-figure 3. These experiments were carried out in the presence of the appropriate one of the following buffers: acetate, tris, or borate, each at a final concentration of 0.02 *M*. The *p*H optimum for the reaction derived from a series of such experiments carried out in the saline solution was about 7.2.

Nature and specificity of the dephosphorylating activity.—Several possible reactions[3] might be suspected in the hydrolysis of ITP: (1) ITP→IDP+P; (2) ITP→IMP+P-P; (3) IDP→IMP+P; (4) 2 IDP⇄ITP+IMP. The identification of the actual reactions occurring was approached first by the estimation of the amount of phosphate liberated in the reaction with a measured quantity of ITP. For this, the ITP in distilled water (8.0 micromoles of intact ITP in 1.0 ml.), prepared as described above, was added to 0.1 ml. undiluted virus preparation that had been suspended previously in the optimal saline medium at *p*H 7.2. The reaction was allowed to proceed to completion at 30° C. The time required for complete hydrolysis under these conditions had been determined before in an analysis of the course of the reaction. After deproteinization with perchloric acid, 0.5 ml. aliquots were removed, and phosphate was determined

[3] ITP=inosinetriphosphate; IDP=inosinediphosphate; IMP=inosinic acid; P=inorganic or orthophosphate; and P-P=inorganic pyrophosphate.

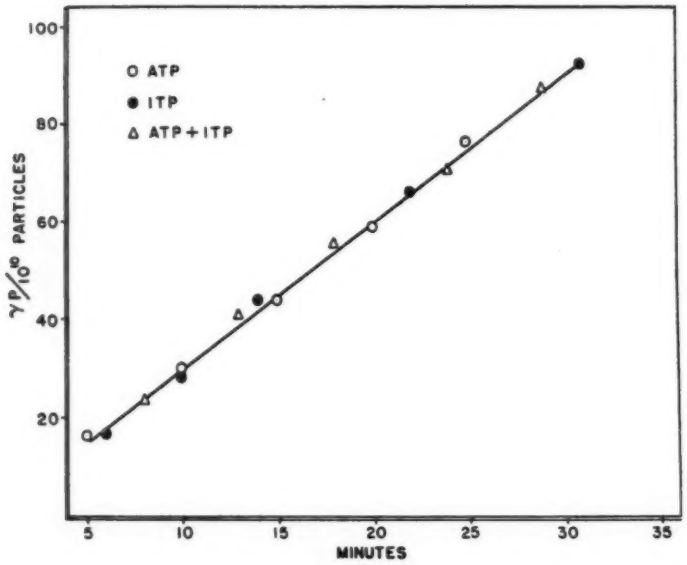
by the method of Fiske and Subbarow (*13*). The quantity of phosphate produced, 7.7 micromoles (after subtraction of the phosphate contributed by the substrate blank), corresponded to 96 percent of the theoretical yield from complete hydrolysis of only the terminal phosphate of ITP.

An aliquot of the same deproteinized reaction mixture was adjusted to *p*H 8.5 with KOH, the preparation was cooled to 4° C., and the precipitated potassium perchlorate was removed by centrifugation. The supernatant fluid was diluted and passed over a column of Dowex-1 (chloride cycle), and the respective nucleotides were successively eluted with the solvents employed in the Cohn and Carter technique (*11*). Quantitative estimation of the individual nucleotides was made with the Beckman spectrophotometer. In addition, a complete spectrum was obtained for each fraction containing measurable nucleotide. Only a trace of the ITP was found. More than 98 percent of the nucleotide content of the preparation consisted of IDP, an amount in close agreement with the molar equivalent of the phosphate already determined in the first series of aliquots. The absorption spectrum of the diphosphate fraction showed a sharp maximum at 2,500 Å, indicating no change in the nature of the hydroxyl group on the purine nucleus. The results of these experiments demonstrated that the only reaction consisted in quantitative hydrolysis of the terminal phosphate of ITP. There was no complicating hydrolysis of IDP, nor was IDP dismuted by the intervention of myokinase.

A comparison of the ITPase and ATPase activities.—Both nucleotides were prepared separately so that each milliliter contained 8.0 micromoles. All experiments were carried out with 10-λ samples of the same virus preparation at *p*H 7.2 (0.02 *M* tris buffer) in the presence of the optimal ionic environment in a final volume of 5 ml. Each experiment was performed in the presence of 8.0 micromoles of nucleotide. In the case of the mixture, ITP+ATP, 0.5 ml. of each was combined, and the resulting milliliter was used to initiate the reaction. Each study of the course of reaction involved a series of identical reaction solutions treated in like manner except for termination of incubation at different times after the start of the hydrolysis by the addition of 2 ml. of 25-percent trichloroacetic acid. Subsequent treatment and phosphate determination were identical with the procedure described above. Text-figure 4 shows that ATP and ITP were hydrolyzed at identical rates. Furthermore, there were no anomalous results when both substrates were present at the same total nucleotide concentration employed when either ATP or ITP was hydrolyzed separately. It appears that the enzyme cannot distinguish between ATP and ITP.

Discussion

The action of the enzyme with respect to ITP is specific and limited, *in vitro*, to the hydrolysis of the terminal phosphate of this substrate. The hydrolysis closely approaches the theoretical endpoint, yielding free phosphate and IDP in equivalent amounts, the latter being unaffected



TEXT-FIGURE 4.—Dephosphorylating activity of the concentrated virus of erythromyeloblastic leukosis on ATP, ITP, and a mixture of the substrates. The mixture contained equal molar parts of the two nucleotides, and the total concentration was the same as that of each separately.

either by hydrolysis or dismutation. In these respects the enzyme behaves in identical fashion toward ITP and ATP (*8*).

As in the case of ATP (*8*), the activity of the enzyme toward ITP is strongly influenced by the nature and concentration of the ions present, and the influence of *p*H is definite and large at optimum monovalent and divalent ion concentration. While the general patterns of these effects for the two substrates are quite similar, there are some significant differences. The ITPase shows maximal activity in the presence of approximately 0.05 to 0.10 molar Na^+ or K^+, a range which coincides with that optimal for the effects of the monovalent ions on ATPase activity. In addition, both sets of curves are closely symmetrical. However, whereas Na^+ has a somewhat greater effect than K^+ in the hydrolysis of ITP, the reverse is true when ATP is the substrate (*8*). Furthermore, the differences between the two ions are greater in the case of ITP hydrolysis. In considering Ca^{++} and Mg^{++} activation, there are noted the same sigmoid curves with both substrates when activity is plotted against divalent ion concentration. With ITPase, however, the optimum occurs at about 10^{-3} *M*, whereas for ATPase it is at approximately 10^{-2} *M*. Also, in the region of the optimal divalent ion concentration, Ca^{++} causes a 20-percent greater activation than Mg^{++} with the former, whereas with the latter Mg^{++} results in a 10-percent greater activation than Ca^{++}. These subtle differences in the responses of ATPase and ITPase related to

Na^+, K^+, Ca^{++} and Mg^{++}, are not unexpected when one recognizes, first, the varying abilities of nucleotides to form complexes with these ions (*14–16*); and, second, by analogy from the findings with myosin ATPase (*14*) and the particulate Mg^{++}-activated ATPase isolated from disintegrated mitochondria (*17*) that the active enzymic intermediate probably involves a ternary complex including substrate, ion, and enzyme.

These findings suggest the participation of only one enzyme in the hydrolysis of ATP and ITP. Further evidence of this is provided by the fact that ATP and ITP are hydrolyzed at identical rates and that the same rate was observed with a mixture containing these two nucleotides at a total concentration equal to that used when the hydrolysis of each substrate was studied separately. These results imply not only that the rates of splitting of ATP and ITP are the same but also that the inhibition caused by ADP and that by IDP are the same in both reactions. In work now in progress (*18*), it has been observed that the inhibition caused by ADP in the hydrolysis of ATP is of the competitive type. As a consequence, it can be assumed, with reasonable assurance, that ADP acts competitively in ITP hydrolysis as does IDP in the hydrolysis of ATP or ITP. Hence, it would appear that the same active centers bind ATP, ITP, ADP, and IDP.

In the earlier studies (*8*) of the hydrolysis of ATP, such conditions were established so that with the concentration of ATP employed there was essentially complete saturation with substrate of the active centers of the enzyme. In view of the observations presented here and the conclusions drawn from them, it appears likely that the present experiments were also carried out under similar conditions of saturation with ITP at the same concentration of nucleotide. It is possible, however, that a complete kinetic study would uncover differences in the nature of rate *versus* substrate concentration curves at levels of substrate considerably below that used in the present experiments. It has already been evident that the virus adenosinetriphosphatase in its specificity, ion activation, *p*H optimum of activity, stability, and other properties is unique among the known analogous enzymes which have been isolated from normal tissues of diverse origin. In the present paper another difference has been demonstrated in addition to those noted above, namely, that ATP and ITP are hydrolyzed at the same rate. In all other cases thus far investigated it has appeared that, under conditions where the enzyme is probably saturated with substrate, such has not been the case (*19–24*).

Particularly interesting with respect to the properties of the virus enzyme are the recent observations by Novikoff *et al.*, who studied, among other things, the activating effect of Ca^{++} and Mg^{++} on the ATPase activity associated with various subcellular fractions isolated from normal liver homogenates by centrifugation. They found that at *p*H 7.4, Mg^{++} stimulated to a greater degree than Ca^{++} in all fractions, the Mg^{++}/Ca^{++} activation ratio being about 2.5 in the mitochondrial and microsomal fractions (*25*). Essentially the same findings were obtained with regenerating and fetal liver fractions (*26*). In striking contrast

with the results of these studies with normal tissue, however, were the observations with liver tumors and other tumors of the rat. In all the tumor materials studied, Ca^{++} and Mg^{++} were equally effective in stimulating the dephosphorylation of ATP (*27*). Furthermore, whereas most of the ATPase activity, both in the presence and absence of the divalent ions, occurred in the mitochondrial fraction of normal adult and in regenerating and fetal liver tissues, in tumors it appeared in the fraction containing the microsomes. Kielley and Kielley have shown that the ATPase activity of the microsomal fraction is limited essentially to the hydrolysis of the terminal phosphate of ATP (*28*). It would appear, therefore, that within the limits of the investigations thus far made there occur major differences between the properties of the ATPases derived from normal tissues and those from tumors, and in the particulate distribution of the enzymes as well. The fact that the ATPase of the virus of erythromyeloblastic leukosis, which produces neoplasia, resembles the enzyme obtained from tumor tissue more closely than the enzyme from normal tissue may be found ultimately to be of more than incidental significance.

Summary

It has been observed that preparations containing the virus of erythromyeloblastic leukosis possess the property to hydrolyze inosinetriphosphate. As in the case of the dephosphorylation of adenosinetriphosphate, the inosinetriphosphatase activity is stimulated by Na^+, K^+, Ca^{++}, and Mg^{++} particularly by the divalent ions; and both enzymes exhibit greatest, activity at the same *p*H, about 7.2. The activity was limited to hydrolysis of the terminal phosphate, and the rate was the same for both enzymes under identical conditions. Both ATPase and ITPase differ in various properties from the analogous enzymes from normal tissue and show greater similarities to those from tumor tissues.

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On the Enzymic Nature of Mitochondrial Characterization by Janus Green B and the Detection of Krebs-cycle Dehydrogenases with Janus Green B[1]

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Janus green B specifically characterizes mitochondria in living cells when these particulates can be observed microscopically to stain, destain, and restain in the absence of staining of other cell components (*1*, *2*). The dye remains oxidized in the presence of oxygen but is reduced first to leuko-Janus green B and then to diethylsafranin and leukosafranin by reducing agents, such as isolated dehydrogenase systems (*3–5*). In living cells, the dye can be maintained in the oxidized state by the cytochrome system, while reduction can occur by the activity of dehydrogenases. In a previous paper (*2*), criteria for mitochondrial characterization in living cells were presented, as well as certain conditions under which isolated liver mitochondrial fractions reduced Janus green B solutions. Staining and destaining of isolated liver mitochondria by Janus green B were not observed.

The present paper describes experiments in which Janus green B was used as a supravital stain for mitochondria *in vitro* and as a hydrogen transfer system with certain dehydrogenases from the Krebs cycle, which is characteristic of mitochondria. Conditions are described under which isolated liver, as well as brain mitochondria, take up and reduce the dye. In addition, the dye was employed as a hydrogen transfer system to study the presence of dehydrogenases in supernatants. Morphology, dye-reducing and enzyme activities, as well as chemical composition of the tissue fractions under varying conditions, are related to the staining reaction and the intactness of the isolated mitochondria.

Materials and Methods

Liver and brain of normal nonfasted mice of the Webster strain were used as materials for staining and for metabolic studies. Entire organs were collected in ice-cooled media and homogenized in a pyrex tube fitted with a plastic plunger. The subcellular fractions were obtained by selective centrifugation of the homogenate (about 20% on a fresh volume

[1] Received for publication February 21, 1955.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

basis, unless otherwise noted) in a refrigerated centrifuge as follows: a 90-second clearance run (maximum r.c.f. ca. 7,000 × g) to remove nuclei and cell fragments and yield a cleared homogenate, followed by centrifugation for 15 minutes at 25,000 × g to yield a mitochondrial pellet and a supernatant with submicroscopic particles. Supernatant fractions were further centrifuged for 45 minutes at 25,000 × g to yield a submicroscopic particle pellet and a supernatant 2. (Modifications are noted in the text). The pellets were then resuspended in media to the volume of the cleared homogenate to make the mitochondrial and submicroscopic particle fractions. Extracting media included: distilled water, 0.25 *M* sucrose, 0.25 *M* sucrose with 10-percent digested gelatin (Knox), 0.88 *M* sucrose, 0.15 *M* NaCl, and a sucrose-salt solution.[3] All media were adjusted to a *p*H of about 7.6 with KOH. Janus green B was prepared in the media at final concentrations of 0.01 to 0.001 percent [percentage expressed as grams of Coleman Bell dye product (CP_3) in 100 ml. solution]; the dye contained approximately 50-percent Janus green B based on nitrogen content. This lot of dye, which was the same as that used previously (*2*), readily and reversibly stained mitochondria supravitally. It was, therefore, suitable for the studies on the mechanism of the staining reaction presented here.

When Janus green B was added to a suspension, the observed color was due to dye both in solution and also bound to particulate material. Sorption of dye was observable microscopically only when sufficient dye had been taken up by the dye-sorbing loci on the particles to contrast them against the pale-blue background of the dye solution. For instance, a homogenate appeared macroscopically blue, but microscopically the particulates were colorless, due to either insufficient chromophilic loci, inadequate sorption of dye, or the presence of the dye in a leukoform. The autoxidizable leukoforms resulted from the action of reducing agents, which donate hydrogen to the dye. The blue oxidized form is converted first to colorless leuko-Janus green B, then to red diethylsafranin and to leukosafranin. These changes occurred both in dye solutions and also when the dye was taken up by particulates. Since, under the present experimental conditions, the dye was reduced through dehydrogenase activity, comparison of the amounts of diethylsafranin formed by the action of specific dehydrogenase systems and by endogenous controls provided a measure of the dehydrogenase activity of the enzyme systems under study. The specific enzyme systems involved were determined by the addition of specific substrates under known conditions. Leukosafranin when formed autoxidized and was measured as diethylsafranin. The terms "coloring" and "color change" will be used in a general sense to describe dye-reduction observed *macroscopically* by changes in the color of solutions, suspensions, and pellets (blue to pink and mixtures of both). Staining will be used to describe *microscopic observations* of particulate-bound dye. Destaining will refer to microscopic observations of par-

[3] Solution contained: KH_2PO_4, 0.003 *M*; K_2HPO_4, 0.008 *M*; KCl, 0.005 *M*; $MgCl_2$, 0.01 *M*; and sucrose, 0.58 *M*.

ticulates which were stained and then appeared colorless when the dye was reduced to a leukoform.

Microscopic staining and destaining.—The Janus green B concentration, as well as the mitochondrial concentration, was varied. The dye was used in concentrations of 0.01, 0.005, and 0.001 percent, with or without glucose, fructose diphosphate, and the appropriate cofactors.[4] Mixtures of 0.1, 0.5, or 0.8 ml. of the original mitochondrial suspension and the appropriate amount of medium, with or without cofactors and substrate, were shaken, and dye was added to obtain the desired concentrations of each component in 0.9 ml. volume. Tubes containing the preparations were kept in ice baths. A drop of the suspension was then placed on a microscope slide, a coverslip added, and the preparation observed immediately through an oil-immersion objective. For comparison, medium containing dye and desired substrate were added directly to a mass of mitochondria taken from the centrifuged nonresuspended pellet and placed on a slide, the method generally used in the previous paper (*2*).

Macroscopic color changes.—Glucose and fructose diphosphate utilization were determined manometrically and by chemical lactic acid analysis [modified Barker-Summerson method (*6*)]. All preparations, including those in the Warburg vessels, were held at approximately 0° C. until immediately prior to starting the experimental run on the Warburg apparatus at 38° C. The aerobic manometric experiments were allowed to run aerobically for 1 hour, unless otherwise noted. Janus green B was then tipped in from the side arm, mixed with the contents of the vessel, and the preparations allowed to stand at 38° C. without shaking for varying periods. Under these conditions, the autoxidation of leukoforms was decreased, and tissue preparations with sufficient dehydrogenase activity became progressively less aerobic. The color of the solution in the vessels was noted as an index of dye reduction[5], that, in turn, indicated the degree of dehydrogenase activity. In some experiments, 95-percent ethyl alcohol was added to the contents of the vessels, and samples were prepared for spectrophotometric determinations of safranin formation as described previously (*2*).

Chemical analyses.—Total nitrogen and total phosphorus were determined by the usual procedures (*7*, *8*).[6] Nucleic acids were extracted with perchloric acid according to the method of Schneider *et al.* (*9*). Ribonucleic acid (RNA) was determined by the method of Mejbaum (*10*), deoxyribonucleic acid by the method of Dische (*11*), and nucleic acid phosphorus according to the usual procedure (*7*). Yeast nucleic acid (Nutritional Biochemicals Corporation) was used as a standard for the

[4] Cofactors contained: adenosinetriphosphate 0.002 *M*, diphosphopyridine nucleotide 0.0007 *M*, niacinamide 0.04 *M*, adenosinediphosphate (sodium) 0.003 *M*, sodium succinate 0.001 *M*, and K_2HPO_4 0.01 *M* (0.05 *M* was used in experiments with fructose diphosphate). Added substrates were: glucose 0.04 *M* or fructose diphosphate 0.007 *M*. Molarities represent concentrations in reaction vessels.

[5] Color descriptions used in the text indicated that the following percentages of reduced Janus green B were present: blue, 0-10 percent; blue (slightly pink), 10-20 percent; blue-purple, 20-40 percent; purple, 40-60 percent; pink (slightly blue), 60-70 percent; pink, 70-100 percent.

[6] The authors are indebted to Mr. Charles Remsburg for the phosphorus and lactic acid analyses and to Mrs. Evelyn Peake for the nitrogen analyses, both of the Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda.

RNA and thymus nucleic acid[7] for DNA determinations. Determinations of nucleic acid by ultraviolet absorption methods were used for comparison. Krebs-cycle intermediates were determined by paper chromatographic methods using phenol : water : formic acid (*12*) and propanol : eucalyptol : formic acid : water (*13*).

Results

Studies with Mouse Liver Preparations

Microscopic staining and destaining of isolated mitochondrial fractions.—When mitochondria were isolated in 0.25 *M* sucrose and the quantity of mitochondria in relation to the concentration of dye was high (*e.g.*, 0.8 ml. of mitochondrial suspension per 0.9 ml. total volume containing 0.005 percent Janus green B), or when mitochondria were smeared directly from the pellet to a drop of dye solution on the slide (*2*), visible staining was not observed. When the proportion of mitochondria to dye was lowered, *e.g.*, 0.1 ml. per 0.9 ml. total volume, staining followed by rapid destaining was observed. Apparently either the dye given was not sufficient to stain large quantities of mitochondria, or the dye-reducing activities of these mitochondria were so high that the dye was reduced before it could be observed. Accordingly, lower proportions of mitochondria to dye were subsequently used to determine the effect of changes in environment upon staining and destaining.

The manner in which the equilibrium between oxidation and reduction of Janus green B was shifted by the addition of certain substrates is illustrated by the data presented in table 1. When only 0.01 percent of dye was added, liver mitochondria stained and destained within 5 minutes. Addition of cytochrome c to the mitochondrial suspension slightly improved the staining but did not appreciably increase oxygen uptake; destaining remained rapid. Addition of sodium succinate further improved staining and increased the oxygen uptake many times, but slowed destaining to 10 or more minutes. Cytochrome c, in addition to succinate, did not seem to improve staining further, but did increase the oxygen uptake; destaining remained slow. Apparently, increasing the activity of the succinoxidase system by the addition of specific substrates enabled the cytochrome system to compete more successfully with the dye for the available electrons, with the result that the dye remained oxidized and the mitochondria stained.

When liver mitochondria were isolated in a sucrose solution of higher molarity (0.88 *M*) and 0.005- or 0.01- percent Janus green B was added (0.1-ml. mitochondrial suspension in 0.9-ml. medium), staining was poor. When the dye concentration was increased to 0.02 percent, staining occurred, but destaining was poor even in the presence of added substrate.

Macroscopic color changes of Janus green B in mitochondrial suspensions.—In the previously reported experiments (*2*) executed under anaerobic conditions with fructose diphosphate as substrate, Janus green

[7] Obtained through the courtesy of Dr. V. E. Price, Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda.

TABLE 1.—*Staining, destaining, and oxygen uptake by isolated liver mitochondria in the presence of added cytochrome c, sodium succinate, and Janus green B**

Addition	Janus green B (0.01 *percent*)		Total μl. O_2 consumed in 35 min.	
	Staining	Destaining (*in minutes*)	Janus green B (*percent*)	
			0	0.005
None	+	<5	18, 11	5
Cytochrome c	++	<5	13, 10	8, 5
Sodium succinate	+++	>10	94, 96	78, 83
Cytochrome c + sodium succinate	+++	>10	108, 105	98, 100
Control + cytochrome c without tissue	——	——	1	+1
Control + cytochrome c + succinate without tissue	——	——	+1	1

*Microscopic observations were made on mitochondrial suspensions prepared by adding 0.1 ml. of mitochondrial fraction to 0.9 ml. of medium that contained additions specified in column 1, and dye.

Reaction vessels contained 0.3 ml. of mouse-liver mitochondria isolated in 0.25 *M* sucrose, plus additions specified in column 1, to give a total volume in main of 1.0 ml. Cytochrome c, 2.4×10^{-4} *M*; sodium succinate, 0.02 *M*. Center well contained 0.1 ml. of 1 *N* KOH. Initial gas phase—air.

B in the surrounding medium was only poorly reduced by liver mitochondrial fractions. Two methods of improving mitochondrial dehydrogenase activity in the presence of fructose diphosphate were tried. First, conditions were supplied for high aerobic metabolism so that a high rate of electron transfer might persist long enough to reduce added Janus green B under subsequent partially anaerobic conditions. Secondly, the concentration of mitochondria used under the conditions just described was increased to make up for enzymes that might be partially lost through leaching.

To test the first condition, a manometric experiment was run for 80 minutes in air. A 20-percent suspension of liver mitochondria, with added cofactors or cofactors plus glucose or fructose diphosphate was used (table 2). Fructose diphosphate increased the oxygen consumption about twofold over that shown by the mitochondria with cofactors only. Janus green B was added from the side arm after 60 minutes, and shaking continued for an additional 20 minutes. The contents of the vessels were then allowed to become partially anaerobic during a final period of 20 minutes. Even the mitochondrial fraction with added cofactors plus fructose diphosphate reduced the dye only to a slight extent. In other similar experiments the mitochondrial fraction occasionally showed a slight reducing capacity but, in general, agreed with results obtained previously under anaerobic conditions; *i.e.*, little or no dye was reduced by the mitochondria.

However, when the concentration of mitochondria was increased by preparation from a 40-percent homogenate, this fraction (table 2), with added cofactors plus fructose diphosphate, reduced the dye in the medium under the conditions described above. This indicated that by increasing the mitochondrial concentrations those dehydrogenases that were able to transfer hydrogen to the dye were increased.

TABLE 2.—*Effect of increasing concentration of mitochondria on the reduction of Janus green B in the presence of added fructose diphosphate (FDP) by liver mitochondria**

Concentration of mitochondrial suspension (*percent*)	No addition		Cofactors		Cofactors+FDP		Cofactors+Glucose	
	μl. O_2 uptake/mg. N in 80 min.	Color	μl. O_2 uptake/mg. N in 80 min.	Color	μl. O_2 uptake/mg. N in 80 min.	Color	μl. O_2 uptake/mg. N in 80 min.	Color
40	33	blue	164	blue (sl. pink)	343	pink (sl. blue)	209	blue
20	9	blue	123	blue	250	blue (sl. pink)	120	blue

*Reaction vessels contained 0.3 ml. of liver mitochondrial suspension plus additions specified (in concentrations given in footnote 4, p. 175), dye, and 0.25 *M* sucrose to give a total volume in main of 0.9 ml. Center well contained 0.1 ml. of 1 *N* KOH. Janus green B added after 60 minutes, color recorded after 100 minutes. Janus green B, 0.005 percent. Initial gas phase—air.

The effect of washing the mitochondria was studied. A mitochondrial pellet was prepared from a 40-percent homogenate, resuspended in fresh 0.25 *M* sucrose, recentrifuged, and suspended again in 0.25 *M* sucrose. In the presence of fructose diphosphate, the unwashed mitochondria reduced the dye in the medium to an extent comparable to that shown by mitochondria in table 2. Corresponding liver supernatants rapidly reduced the dye. The washed mitochondria reduced the dye only slightly, concomitant with reduced metabolic activity. This suggested the instability of liver mitochondria, as the enzymes involved in oxygen consumption and dye reduction were removed to a great extent by only one washing.

Dye reduction by the Recknagel procedure.—Potter, Recknagel, and Hurlbert (*14*) observed Janus green B reduction when the dye was added to homogenates of rat liver, prepared in isotonic sucrose, and stratified by centrifugation. After standing at 38° C., the mitochondrial layer of the pellet reduced the dye, but the upper submicroscopic particle (microsome) layer remained blue.

Similar results were obtained in the present study when Janus green B (0.005 percent) was added to mouse liver homogenate, prepared in 0.25 *M* sucrose, and then centrifuged. The sediment took up most of the dye and was uniformly blue-colored. On standing at 38° C., only the submicroscopic particle layer remained blue (even after 16 hours). The supernatant appeared pink within 15 minutes, while the pellet appeared rose-blue and shortly afterward pink. It should be noted that this procedure indicated that sufficient dehydrogenases were present and active in both supernatant and mitochondria, and not in the "microsomes," to change the color of the dye. Observations obtained by this procedure did not substitute for data from observations of microscopic staining and destaining of the mitochondria. Further, this procedure did not eliminate the possibility of exchange of solubilized enzymes from fraction to fraction.

From the above experiments it can be concluded that isolated liver mitochondria can be observed microscopically to stain and destain under given conditions. It can also be concluded that a suspension of liver mitochondria with high glycolytic activity (*15*) will reduce Janus green B in solution only when the concentration of those dehydrogenases that are able to transfer electrons to the dye is increased by increasing the concentration of the mitochondria.

Liver supernatants, in contrast, very readily reduced the dye.

Studies with Preparations of Mouse Brain

Microscopic staining and destaining.—Mitochondrial suspensions of mouse brain in 0.25 *M* sucrose in amounts of 0.1 ml. and 0.5 ml. per 0.9 ml. of medium clearly stained blue with 0.005- or 0.01- percent Janus green B. These preparations destained slowly after 10 minutes with 0.005 percent, and more slowly, or faintly, with 0.01 percent.

When higher proportions of mitochondria, *e.g.*, 0.8 ml. in 0.9 ml., were used, visible staining was not observed unless the concentration of dye

used was increased. As in the case of liver, mitochondrial smears taken directly from pellets and placed in a drop of dye on the slide frequently failed to show staining. However, when only a small amount of mitochondria was used, staining was observed by this method. These observations showed that absence of microscopic staining can be due to low dye concentration in relation to tissue concentration.

Addition of glucose as substrate to the mitochondrial suspensions led to destaining of the particulates within 5 minutes. Apparently, the activity of those dehydrogenases that are able to transfer electrons to the dye was increased on addition of substrate.

Brain mitochondria prepared in 0.88 *M* sucrose media in amounts of 0.1 ml. per 0.9 ml. medium clearly stained blue with 0.005- and 0.01- percent dye. In the absence of substrate they destained poorly, or not at all, within 10 minutes, the high sugar concentration apparently decreasing dehydrogenase activity. In the presence of added substrate fructose diphosphate and cofactors, the mitochondria stained with the lower dye concentration and destained within 10 minutes.

Morphology, staining and dye-reducing activities of brain fractions in various media.—It was desirable to further clarify, in terms of the enzyme activity found, the staining and destaining phenomena shown by mitochondria in living cells. Brain fractions provided suitable material for such a study because of the distinct staining reactions shown by the isolated mitochondria, and the distribution, after extraction, of certain enzyme systems (*e.g.*, Krebs cycle in the mitochondrial and the glycolytic system over both supernatant and mitochondrial fractions). Glucose was selected as a substrate, since it was rapidly metabolized by brain fractions and hence a good source of electrons. Several media were compared, including 0.25 *M* sucrose, 0.58 *M* sucrose with salts, 0.15 *M* NaCl, and distilled water.

In preparing the fractions, it was observed that in 0.25 *M* sucrose the nuclear fraction was heavily contaminated with mitochondria but the mitochondrial fraction was practically free of nuclei. In 0.15 *M* NaCl and distilled water the separation of a fraction containing most of the nuclei was not readily obtained. In these two media, visible particulate material settled even at less than 2,000 × g for 45 seconds.

Table 3 shows the results obtained with the subcellular fractions. The morphologic appearance of the fractions, observed with a dark phase-contrast microscope and an oil-immersion objective, is given in column 3 of the table. It can be seen that the greatest morphologic deterioration of the mitochondria occurred in distilled water and the least in 0.58 *M* sucrose with salts. Brain mitochondria deteriorated, as did mitochondria of other origin, from uniformly refracting globular or elongated bodies, to globular bodies with strongly refracting material appearing as a crescent, to disintegrated granular material; see also Zollinger (*16*).

Results from microscopic observations of staining are shown in columns 4 and 5. Mitochondria stained when prepared in all media except distilled water, where dye sorption by the mitochondria was slight or doubtful.

TABLE 3.—*Glucose utilization and Janus green B reduction by mouse brain fractions in several media* *

Medium	Fraction †	Appearance	Janus green B		Q_{O_2}		Q_{l-a}‡		Solution color	
			Stain	De-stain§	Co-factors	Cof.+ glucose	Co-factors	Cof.+ glucose	Co-factors	Cof.+ glucose
0.25 *M* sucrose	N	Optically empty	0	—	—	—	—	—	—	—
	M	Most rounded, some partly elongated.	+	+	68	173	129	709	purple	pink
	SN	Opaque			17	20	370	2225	blue	blue
	M + SN	—			83	159	114	2395	purple	pink
0.58 *M* sucrose+salts‖	N	Optically empty	++		—	—	—	—	—	—
	M	Partly elongated and rounded	++	+	40	142	151	770	purple	pink
	SN	Opaque			7	7	667	2670	blue	blue
	M + SN	—			73	146	287	2380	blue-purple	pink
0.15 *M* NaCl	N	Granular	++		—	—	—	—	—	—
	M	Rounded, crescents	++	?	55	91	84	330	purple	pink
	SN	Clear			16	20	285	2060	blue	blue
	M + SN	—			53	79	44	730	purple	pink
H_2O	N	Optically empty, except nucleoli.	+		—	—	—	—	—	—
	M	Advanced crescents	?	?	36	89	26	233	blue	purple
	SN	Clear			15	22	84	2410	blue	blue
	M + SN	—			50	104	51	2375	purple	purple

*Reaction vessels contained 0.2 ml. of each mouse brain fraction specified, plus additions noted in concentrations given in footnote 4, and dye and medium to give a total volume in the main of 0.9 ml. Center well contained 0.1 ml. of 1 *N* KOH. Janus green B added from the side arm after 60 minutes. Color recorded after 17 hours. Janus green B, 0.005 percent. Initial gas phase—air.

†N=nuclei, M=mitochondria, SN=supernatant.

‡Q_{l-a}=*gamma* lactic acid formed per mg. nitrogen per hour, duration of experiment—90 minutes.

§Within 10-minute period in presence of glucose and cofactors.

‖*Cf.* footnote 3, p. 174.

In 0.25 *M* sucrose the mitochondria generally appeared selectively stained, while, in the other media, nuclei also frequently stained. Destaining occurred in the presence of added substrates, in general only in 0.25 *M* sucrose and in 0.58 *M* sucrose plus salts media. Whenever the mitochondria had progressed to the crescent state, the dye, when observable, appeared to be concentrated in the crescent.

In all experiments the mitochondria and mitochondrial plus supernatant fractions utilized glucose, as measured by increases in oxygen consumption and amount of lactic acid formed, in comparison with the corresponding endogenous fractions. Supernatant fractions did not oxidize glucose, but produced large amounts of lactic acid. In 0.25 *M* sucrose and in 0.58 *M* sucrose plus salts, glucose oxidation by the mitochondrial fraction was approximately twice that obtained in mitochondrial fractions isolated in saline or distilled water. Likewise, the mitochondrial fraction in sucrose formed over twice as much lactic acid as in saline, and three times that formed in distilled water.

Reduction of Janus green B was clearly dependent on the magnitude of activity of the dehydrogenases of the mitochondrial fraction, as expressed by oxygen uptake. In 0.25 *M* sucrose, for example, visible reduction (color change) was observed within 30 minutes after adding the dye to vessels containing mitochondria and mitochondria plus supernatant. The color changes given in the table were recorded after 16 hours and, therefore, do not reflect accurately the rate of dye reduction by the fractions with or without substrate. The vessels containing supernatant, however, remained blue even after 16 hours, notwithstanding a high activity of the glycolytic dehydrogenases. At this time, the mitochondria and mitochondrial plus supernatant suspensions with added cofactors and glucose appeared pink, and with added cofactors alone appeared purple. It should be noted that the supernatant also failed to reduce the dye both in media containing added electrolytes and in distilled water. Fractions isolated in 0.25 *M* sucrose plus 10-percent gelatin were similar in behavior to those isolated in the sucrose-salt medium. Metabolic data have been omitted since the nitrogen content of the gelatin prevented calculation of the Q values.

It was apparent that, under these conditions, dye reduction occurred only where mitochondrial dehydrogenases could function.

Dye reduction by the Recknagel procedure.—Janus green B (0.005 percent) was added to mouse brain homogenates prepared in 0.25 *M* sucrose, and the preparation centrifuged. Results differed from those obtained with liver homogenates in that the dye remaining in the supernatant was not reduced. Macroscopically, the pellet was uniformly colored blue and after standing, the top layer of the pellet containing the submicroscopic particles remained blue, whereas the mitochondrial layer became red. The results are similar to those obtained with the corresponding fractions in the manometric experiments previously described.

Experiments carried out in a nitrogen atmosphere.—Experiments under anaerobic conditions were performed to determine whether the reducing

activities of brain fractions would be different from those described above under conditions which were more favorable for dye reduction. Glucose with appropriate cofactors was added as substrate for reactions run in an atmosphere of nitrogen. Results (table 4) agreed with those obtained when reactions were run under initially aerobic conditions as described above. Mitochondrial fractions reduced the dye, the supernatant fraction did not. In addition, it was shown that mitochondria which had been washed once, especially with added substrate, readily brought about safranin formation. The submicroscopic particle fraction, separately or in combination with the supernatant, failed to reduce the dye within 1 hour.

TABLE 4.—*Reduction of Janus green B by mouse brain fractions under anaerobic conditions**

Fraction†	N, mg. per vessel	Percent safranin formed	
		Cofactors‡	
		No added substrate	Glucose
H	0.52	64	73
M	.28	45	90
wM	.23	20	72
mW	.05	0	0
SN_2	.14	0	0
X	.01	0	0
$M+SN_2$	.37	55	87
$X+SN_2$	.10	0	0
wM+mW	.28	38	100
Control without tissue	—	0	0

*Reaction vessels contained: 0.2 ml. of each mouse brain fraction specified plus cofactors and substrate noted (in concentrations as given in footnote 4), dye and 0.25 *M* sucrose medium to give a total volume in main of 0.9 ml. Janus green B added from side arm after 10-minute equilibration period. Dye reduction determined after 90-minute experimental run. Janus green B: 0.01 percent. Initial gas phase—N_2.

†H=homogenate, M=mitochondria, wM=washed mitochondria, mW=supernatant from washed mitochondria, SN_2=supernatant 2, X=Submicroscopic particles.

‡Without succinate.

Studies on the dye-reducing capacity of mouse brain supernatants.—The previous experiments left unanswered the questions of whether brain supernatants and, in particular, the glycolytic dehydrogenases which contributed to high lactic-acid production in this fraction had the ability to reduce Janus green B under any conditions. Favorable conditions were provided by using lower dye concentrations, by limiting the oxygen supply, and by increasing the proportion of fraction to medium in the reaction vessel. A 20-percent mouse brain homogenate prepared in 0.25 *M* sucrose was cleared of nuclei and divided into 2 aliquots. Janus green B was added to one portion (A) before and to a second portion (B) after centrifugation at 25,000 × g for 45 minutes. The dye in the supernatant of A, most of which was sedimented with the particulates, was replaced.

Table 5 shows that under anaerobic conditions Janus green B at a concentration of 0.0025 percent was not reduced by supernatant without added substrate or cofactors, very slowly reduced by supernatant with

TABLE 5.—*Anaerobic reduction of Janus green B by mouse brain supernatants**

Homogenate from which supernatant was derived		Additions to SN_2†		Color after 30 minutes	Color after 2 hours
Portion	Janus green B (*percent*)	Janus green B (*percent*)	Other		
A	0.0025	0.001	0	blue	blue
			Cofactors	blue	pink
			Cofactors+FDP‡	pink (sl. blue)	pink
B	0	0.0025	0	blue	blue
			Cofactors	blue (sl. pink)	pink
			Cofactors+FDP‡	pink (sl. blue)	pink

*A 20-percent mouse brain homogenate (by volume) prepared in 0.25 *M* sucrose was cleared of nuclei and divided into 2 aliquots. Dye was added to one portion (A) before, and to another portion (B) after centrifugation at 25,000 × g for 45 minutes. Additional dye was then supplied to the supernatant from A, to replace the dye which was taken up by the particulates. Aliquots (1.8 ml.) of supernatants from A and B were then pipetted into test tubes. Cofactors with or without substrate (in concentrations given in footnote 4) were added to make the final volume 3.0 ml. The air in the test tubes was replaced with 95-percent nitrogen and 5-percent carbon dioxide, and the tubes were stoppered and held without shaking at 38° C.

†SN=supernatant.

‡FDP=fructose diphosphate.

cofactors, and reduced somewhat faster by supernatant with substrate and cofactors. Supernatant from aliquot A, which contained the dye before centrifugation, reacted somewhat more slowly than the supernatant from aliquot B, indicating that the Janus green B might have had a precipitating effect on previously solubilized dehydrogenases during the centrifugation. The reduction by the mitochondria from aliquot A, on the other hand, was very rapid; the dye was already reduced in the mitochondrial pellet during centrifugation. Comparable results were obtained in similar experiments run in Warburg vessels (0.001-percent Janus green B). These experiments were, however, initially aerobic and became progressively anaerobic after shaking was stopped. The mitochondrial fractions appeared pink within 30 minutes, whereas brain supernatants remained blue after 1 hour. After continuation of the experiment overnight, the supernatants finally appeared pink. At higher dye concentrations the supernatant remained blue. These experiments showed that brain supernatants could reduce Janus green B at a slow rate only under special conditions (low dye concentration, anaerobiosis), notwithstanding high glycolytic dehydrogenase activity (high lactic-acid formation). Whether the safranin formation observed was due to the activity of glycolytic or one or more Krebs-cycle dehydrogenases, activated by the addition of fructose diphosphate, was not yet evident.

Janus green B as a hydrogen transfer system in brain and liver supernatants.—The experiments reported thus far have shown that Janus green B was rapidly reduced by liver supernatants and not by brain supernatants, except under special conditions, notwithstanding similar high glycolytic activity in both types of supernatants (*15*, *17*).

These results indicated that there was no direct relation between the degree of activity of the glycolytic dehydrogenases by themselves and the degree of reduction of Janus green B. They suggested that the glycolytic dehydrogenases could not transfer hydrogen to Janus green B and that the difference in dye-reducing activity between liver and brain supernatants might be due to a difference in amount of dehydrogenases of another type in these supernatants, most likely those of the Krebs cycle.

Earlier experiments employing Krebs-cycle substrates, demonstrated some Krebs-cycle dehydrogenase activity in terms of increased oxygen consumption in liver supernatant, but not in brain supernatant. This implied the presence of the cytochrome system, at least in limited amounts, in liver supernatants. The virtual absence of cytochrome oxidase from brain supernatants was demonstrated by the absence of oxidation of *p*-phenylenediamine that had been added to this fraction.

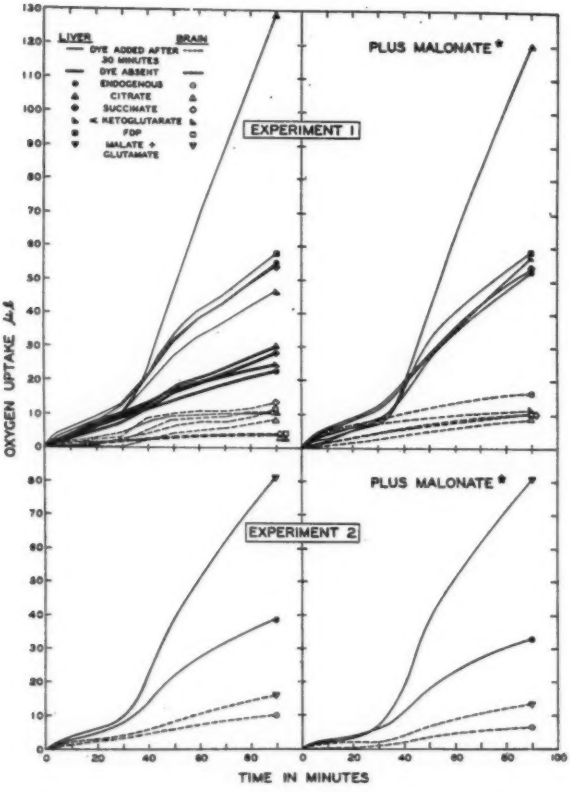
This left two possibilities open to explain the difference in dye reduction shown by liver and brain supernatants: 1) either the Krebs-cycle dehydrogenases tested for were present in liver supernatants and absent from brain supernatants (in which case the weak dye reduction by the latter would have to be attributed to the glycolytic or other unknown dehydrogenases), or 2) such dehydrogenases were present in both supernatants but functionally inactive in the brain fraction, because of the absence here of the cytochrome oxidase system. This latter alternative suggested the use of an artificial oxidase. Since it was known that certain dyes functioned as "oxidases" or hydrogen transfer systems between dehydrogenases and molecular oxygen (*18*, *19*), it was decided to study the effectiveness and specificity of Janus green B in this respect.

The dye was added to brain or liver supernatants containing appropriate cofactors with or without substrates, with air as the gas phase. Text-figure 1 presents the results.

Reaction vessels contained: 0.3 ml. of each SN_2 fraction specified, plus cofactors (in concentrations as given in footnote 4), substrates, dye as noted, and medium to give a total volume of 0.9 ml. in the main. Janus green B, 0.1 μM; sodium succinate, 0.02 M; potassium citrate, 0.02 M; malic acid, 0.02 M; and α-ketoglutaric acid, 0.02 M. Janus green B added from side arm after 30 minutes. Center well contained 0.1 ml. 1 N KOH. Initial gas phase—air.

Liver supernatants without added substrate showed a measurable oxygen uptake. The addition of dye to such preparations increased this endogenous oxygen uptake. The further addition of substrates, such as succinate, α-ketoglutarate, or fructose diphosphate did not appreciably improve oxygen utilization, although with fructose diphosphate the usual large amounts of lactic acid were formed. However, preparations to which citrate was added showed a further increase in oxygen uptake, following the addition of the dye; with malate a smaller increase was noted.

These data indicated the presence of malic and citric dehydrogenases in liver supernatants, and the function of Janus green B as a hydrogen transfer mechanism between these dehydrogenases and molecular oxygen.



TEXT-FIGURE 1.—Janus green B as a hydrogen transfer system in mouse brain and liver supernatant fractions.

These two observations were corroborated by paper chromatography of the preparations. In the presence of dye with citrate as substrate, the citrate spot decreased in size and a strong α-ketoglutarate spot appeared. With dye and malate, the malate spot decreased and a strong lactate spot appeared. The presence of lactate was confirmed by the chemical test with *o*-phenylenediamine and by lactic-acid determination.

Brain supernatants, without added substrate, showed a negligible oxygen uptake. When dye was added, the oxygen uptake was only very slightly increased. The further addition of succinate, α-ketoglutarate, citrate, or fructose diphosphate did not appreciably alter the oxygen uptake, although large amounts of lactic acid accumulated with fructose diphosphate as substrate. Only when malate, with or without glutamate, was supplied as substrate, did addition of dye cause a slight increase in oxygen uptake, indicating the presence of some malic dehydrogenase. The activity of this trace of malic dehydrogenase might account for the slight Janus green B reduction by brain supernatants under the special

conditions of table 5, if the endogenous preparation or the added fructose diphosphate contained traces of malate.

From these data it can be concluded that liver supernatants contained appreciable amounts of citric and malic dehydrogenase. Conversely, since under similar conditions no appreciable increase in oxygen uptake was measured with brain supernatants, it can be concluded that the Krebs-cycle dehydrogenases tested were essentially absent from this fraction except for traces of malic dehydrogenase.

The dye reacted in neither supernatant in conjunction with glycolytic dehydrogenases, notwithstanding the fact that the large lactic-acid accumulation demonstrated considerable activity of these dehydrogenases. This lack of reactivity may have been due to the absence of flavine linkages in the nonreactive (paired) glycolytic dehydrogenases.

When similar experiments were performed with brain or liver mitochondria, in all cases addition of the dye resulted in a decrease in rate of oxygen uptake. Since either oxygen transfer system alone, under given conditions, was shown to oxidize measurable amounts of substrate, this interference in the mitochondria might have been due to an interaction between the two systems. That this interference did not take place at the cytochrome oxidase level was shown by the fact that oxidation of *p*-phenylenediamine by liver mitochondria was not decreased in the presence of Janus green B. Only in the absence of the cytochrome system, as in the brain supernatants, is the measurement of the hydrogen-transferring function of the dye in terms of oxygen utilization meaningful. Further experiments will be presented elsewhere.

An interpretation of these results in relation to the use of Janus green B as a specific indicator for mitochondria is presented in the discussion.

A study of the staining properties in relation to chemical composition.—It was pointed out above that the intensity of the staining of mitochondria depended on the number of loci of dye sorption, given an equal rate of destaining. In the previous sections it has been shown that liver supernatants contained more Krebs-cycle dehydrogenases than comparable brain supernatants, suggesting a considerable loss of these enzymes from liver mitochondria. At the same time, these liver mitochondria stained less clearly and destained more rapidly than similarly isolated brain mitochondria. This suggested that liver mitochondria might have lost not only more dehydrogenases, but also more dye-sorbing loci than the brain mitochondria; *i.e.*, they took up less dye than the latter and hence destained faster, although they had fewer dehydrogenases.

Therefore, studies were made to determine whether these differences between brain and liver preparations could be correlated with differences in chemical constitution and perhaps throw some light on the nature of the dye-sorbing loci. Although a number of analyses of subcellular fractions are reported in the literature (*9*, *20–23*), it was necessary to determine whether the fractionation methods here yielded similar results.

Twenty-percent homogenates were prepared in 0.25 *M* sucrose (*p*H 7.6) from equal volumes of tissues of mouse brain and liver. Equal aliquots

TABLE 6.—*Nitrogen, phosphorus, RNA, DNA, and nucleic acid-phosphorus content in milligrams per milliliter of fractions of mouse brain and liver.**

Fraction†	Liver					Brain				
	N	P	RNA	DNA	NA-P‡	N	P	RNA	DNA	NA-P‡
N	3. 16	0. 33	0. 623	0. 522	0. 101	1. 30	0. 24	0. 124	0. 318	0. 044
wN	2. 00	. 21	. 448	. 516	. 080	(.84) §	. 13	. 056	. 282	. 030
nW	1. 05	. 13	. 321	. 024	. 027	. 46	. 105	. 069	. 062	. 009
M	. 69	. 08	. 253	. 021	. 020	1. 32	. 27	. 088	. 047	. 009
wM	. 60	. 075	. 193	. 021	. 014	1. 16	. 24	. 078	. 044	. 008
mW	. 09	. 014	. 040	0	. 003	. 14	. 05	. 028	. 018	. 001‖
SN_2	2. 17	. 21	. 551	. 036	. 049	. 79	. 10	. 136	. 021	. 011
X	. 48	. 08	. 298	. 021	. 020	. 20	. 04	. 044	. 011	. 002
H	6. 27	. 70	1. 626	. 546	. 182	3. 65	. 66	. 330	. 366	. 065

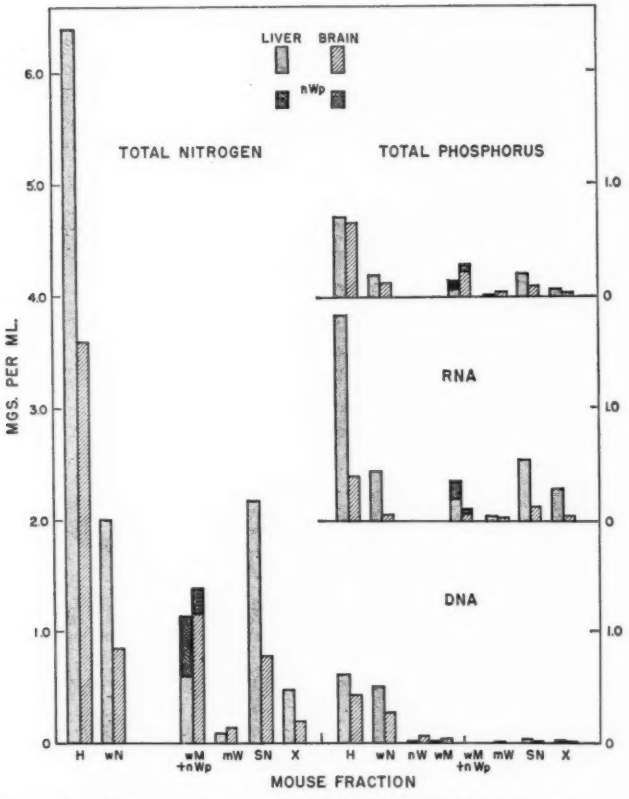
*Fractions of both tissues were prepared from 20-percent (by volume) homogenates in 0.25 *M* sucrose. Nuclei and cell fragments were spun down in a clearance run at speeds up to 2,500 × g for 90 seconds to yield nuclear pellets and supernatant fluids containing M + SN. Nuclear pellets were resuspended in medium to the original volumes of the homogenates to make the nuclear fractions (N), which were again centrifuged at up to 2,500 × g for 90 seconds to yield washed nuclear pellets and nuclear wash supernatants (nW). (Microscopic examination showed that the nW fraction contained many mitochondria.) Aliquots of M + SN fractions were further centrifuged for 15 minutes at 25,000 × g to yield mitochondrial pellets and SN fractions. The mitochondrial pellets were resuspended in medium to their previous volumes to give the M fractions. Aliquots of M and SN fractions were then spun for 45 minutes at 25,000 × g to yield, respectively, washed mitochondrial pellets plus mitochondrial wash supernatants (mW) and submicroscopic particle pellets plus supernatant 2 (SN_2). The remaining pellets were resuspended to their previous volumes to make the corresponding washed nuclear (wN), washed mitochondrial (wM) and X fractions.

†N=nuclei, wN=washed nuclei, nW=nuclear wash, M=mitochondria, wM=washed mitochondria, mW=mitochondrial wash supernatants, SN_2=supernatant 2, X=submicroscopic particles, H=homogenate.

‡NA-P=nucleic acid—phosphorus.

§ Estimated value obtained by substracting nW from N.

‖ Estimated value obtained by subtracting wM from M.



TEXT-FIGURE 2.—Nitrogen, phosphorus, RNA, and DNA distribution in fractions obtained from mouse brain and liver tissues.

Fractions are designated: H=homogenate, N=nuclei, wN=washed nuclei, nW=nuclear wash supernatant, nWp=nuclear wash pellet, M=mitochondria, wM=washed mitochondria, mW=mitochondrial wash supernatant, SN=supernatant, and X=submicroscopic particles.

of each homogenate were centrifuged to yield: nuclei, washed nuclei, supernatant from washed nuclei, mitochondria, washed mitochondria, supernatant 2, and submicroscopic particles. Nitrogen, phosphorus, RNA, DNA, and nucleic acid-phosphorus content of the fractions are presented in table 6. A comparison of the total amounts found in the homogenates of both tissues showed that the liver homogenates contained approximately twice as much total nitrogen, 4 times as much RNA, 1.5 times as much DNA, and nearly the same total phosphorus as the brain homogenates. Analyses of brain and liver homogenates from various animals have also been reported by others (*24–28*).

The distribution of nitrogen, phosphorus, RNA, and DNA over the separated fractions, as shown in the above table, is graphically presented in text-figure 2. When nuclear wash supernatants were further centrifuged

at sufficient speed to separate mitochondria, the nuclear wash pellets, which microscopically consisted mainly of mitochondria, contained approximately half of the nitrogen, phosphorus, and RNA of the nuclear wash supernatant. Accordingly, in the graph, half of the nuclear wash fraction has been considered mitochondria and added to the washed mitochondrial fraction. It is indicated by the shaded part of the bar. In the case of the liver mitochondrial fraction, roughly half of the bar is shaded; in the case of the brain mitochondrial fraction about one fifth. This implies that in the commonly used technique of preparation a significant amount of mitochondrial material remains with the nuclear fraction and is responsible for its metabolic activity, especially in the liver. The DNA data supply some measure of the contamination of the fractions with nuclei and nuclear material. This contamination is inherent to the technique used. Fat droplets and broken-up myelin (brain), present as contaminants in all the fractions, contributed to the total phosphorus and nitrogen values.

It can be seen that liver supernatants contained considerably higher amounts of nitrogen and phosphorus and somewhat higher amounts of RNA than did the liver mitochondria. On the other hand, brain supernatants contained lower proportions of nitrogen and phosphorus and nearly the same amount of RNA as did the brain mitochondrial fraction. Only total phosphorus was found in higher amounts in the mitochondrial fraction from brain than in the mitochondrial fraction from liver, and was largely present in an alcohol-ether soluble form (phospholipid). The submicroscopic particle fraction was, on a volume basis, a small fraction containing, in brain and liver respectively, 5 and 8 percent of the total nitrogen, 6 and 11 percent of the total phosphorus, and 11 and 16 percent of the RNA.

Discussion

Mitochondria in living cells can be identified by Janus green B when the dye is applied in such a way as to readily obtain an oxygen-dependent, reversible staining of the cytoplasmic particulates in the absence of a nuclear or an interparticulate cytoplasmic staining (*1*, *2*). Two views have been expressed in the literature regarding the mechanism of the supravital Janus green B staining reaction.

According to the first view (*2*), the dye is taken up and concentrated rapidly by the mitochondria and not concentrated by the nonparticulate cytoplasm of living cells. The resulting reversible supravital staining of metabolically active mitochondria depends on the equilibrium between the activity of oxidase and dehydrogenase systems, both of which are on the mitochondria (*15*, *17*, *23*, *29*, *30*). Thus, according to this view, mitochondria in living cells contain loci for dye sorption and enzyme systems involved in this staining reaction, both of which are absent in the interparticulate cytoplasm.

According to the second view (*4*, *31–33*), Janus green B is taken up throughout the cell but is more rapidly reduced by the non-mitochondrial portions than by the mitochondria. The mitochondria contain the cytochrome system (*23*, *29*, *34*), which slows reduction of the dye and permits its retention in an oxidized form on these sites. Thus, according to the latter view, loci of dye sorption and dehydrogenase systems would be present throughout the cell.

The following evidence has been presented in support of the first view. In the first place, when Janus green B is added to mitochondria, either in living cells or in fractions, microscopic observation indicates that sufficient loci of dye sorption are present on the mitochondria to concentrate the dye to the extent that staining can be observed.

Second, the mitochondria contain the cytochrome system (*23*, *29*, *34*), which permits the dye to be maintained in an oxidized state on the mitochondria in the presence of oxygen. In the absence of oxygen, or when the cytochrome system is blocked by inhibitors (*1*, *35*), the dye is reduced.

Third, in this paper it was established that Janus green B was reduced through the activity of Krebs-cycle dehydrogenases and not by the glycolytic dehydrogenases. It was established previously that a functioning Krebs cycle can be demonstrated only in the mitochondrial fraction from various tissues (*15*, *17*, *30*), and it was shown in this paper that, in brain, the Krebs-cycle enzymes investigated were in the mitochondrial fraction except for a trace of malic dehydrogenase. It follows that Janus green B reduction is dependent upon enzymes located on the mitochondria. This conclusion was further substantiated by the finding that Janus green B could serve as a hydrogen transfer system with Krebs-cycle dehydrogenases but not with the glycolytic dehydrogenases activated by the addition of fructose diphosphate.

Fourth, the absence of observable staining of the interparticulate cytoplasm in the living cell (*1*, *2*) indicates the absence of dye-sorbing loci, since supernatants from brain, in which this cytoplasm is represented, were demonstrated to lack the dehydrogenase activity necessary to destain the dye.

The results presented lead to the conclusion that Janus green B can thus be used to identify mitochondria in living cells because 1) these organelles contain sufficient dye-sorbing loci to stain collectively with this dye, 2) they alone contain the Krebs-cycle dehydrogenases which will rapidly reduce the dye under the proper conditions, and 3) they alone contain oxidases, so that the dye can be oxidized rapidly when oxygen is introduced and stay oxidized in its presence.

Therefore, *in vivo*, the dye is a specific indicator of mitochondria only when selective staining, destaining, and restaining of particulates have been observed microscopically.

On the basis of the data presented, it is apparent that the dye can be used *in vitro* as a hydrogen transfer mechanism which distinguishes between the activity of Krebs-cycle dehydrogenases and that of glycolytic dehydrogenases. Consequently, it could be used to determine the degree of

preservation of mitochondrial elements by determining the amount of Krebs-cycle dehydrogenases appearing in the corresponding supernatants, even in trace amounts, by measuring the oxygen uptake by supernatants in the presence of the respective Krebs-cycle intermediate and Janus green B. On the basis of the data obtained in this manner, it can be inferred that mouse brain mitochondria lost only a small amount of Krebs-cycle dehydrogenases to the supernatant and, therefore, were isolated in a more intact condition than liver mitochondria.

Summary

The staining and destaining phenomena of mitochondria with Janus green B were duplicated on isolated mitochondria, and their dependence on mitochondrial-bound enzymes and loci of dye sorption was demonstrated.

Janus green B solutions, under the conditions described, were reduced readily by mitochondrial fractions and slightly or not at all by supernatant fractions of mouse brain. In contrast, the dye solutions were rapidly reduced by liver supernatants and only poorly reduced by liver mitochondrial fractions. In these experiments the dye reduction in the medium depended on the distribution of the activity of the Krebs-cycle dehydrogenases among the respective fractions, and not directly on the activity of the glycolytic dehydrogenases.

Brain mitochondria were isolated and studied in various media, each of which led to changes, in varying degrees, in the morphology of the mitochondria, in the staining and destaining of the mitochondria, and in the rate of enzyme activity of the subcellular fractions. Judging from these results, the degree of intactness of the mitochondria was highest in the sucrose solutions.

Adding the dye to mitochondrial preparations decreased the rate of oxygen uptake with respect to the control preparations. Added to supernatants the dye served as a hydrogen transfer system and markedly increased oxygen uptake when Krebs-cycle dehydrogenases were activated, but not when glycolytic dehydrogenases were activated. Of the Krebs-cycle dehydrogenases tested, only citric and malic dehydrogenases were found in appreciable amounts in liver supernatants, and only traces of the latter in brain supernatants.

Under the conditions described, liver supernatant fractions contained higher amounts of nitrogen and phosphorus and slightly higher amounts of ribonucleic acid than corresponding liver mitochondrial fractions. Brain supernatant fractions contained lower amounts of nitrogen and phosphorus and nearly the same amount of ribonucleic acid as brain mitochondrial fractions.

The results in general indicated that isolated brain mitochondria were more intact than liver mitochondria in the medium used.

The data allow the following synthesis: The specific supravital staining of mitochondria in living cells with Janus green B is due to a rapid sorption and concentration of the dye on mitochondrial loci, followed, under the given conditions, by destaining due to the activity of dehydrogenases of

the Krebs cycle, characteristic for these elements. In living cells the interparticulate cytoplasm is devoid of dye-sorbing loci and dye-reducing dehydrogenases, thus limiting the staining and destaining reactions specifically to the mitochondria.

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Comments on the Analysis of Dose-Response Data in Experimental Carcinogenesis[1]

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There has been recent interest in the dose-response characteristic for the production of a particular tumor by a carcinogen (*1–4*). From analyses of such data, investigators interested in the mutation hypothesis for the etiology of tumors hope to obtain estimates of the number of mutations or carcinogen-induced changes required for the transformation of normal cells into potential tumor cells. Although each of the previous studies (*1–4*) makes valuable contributions, the experimental material analyzed does not seem to be quite suitable for this purpose.

It therefore appears desirable to present the following discussion of a simple theory of the occurrence of tumors induced by carcinogens, based on the mutation hypothesis. This discussion attempts to indicate the kinds of data that are needed for the analysis mentioned above and the reasons why available data are not suitable.

Comments on Theory

In attempting to quantitate the mutation hypothesis for the origin of tumors induced by carcinogens, the following analysis is based on analogy with mutation induction in a population of separate cells, each of which may be tested for change such as is done for sperm or bacteria. It should be recognized that the construction of a valid quantitative theory based on the mutation hypothesis is probably not possible at the present time because so little is understood about mutations in general and about somatic mutations in particular. For simplicity, the following three recent observations on mutation induction will be ignored in this analysis: 1) there may be one or more transition states between the normal cell and the manifestation of the properties of the mutant (*5*); 2) in some cases a transition state may be reversible so that the changed cell may revert back to normal (*6*, *7*); 3) in the induction of mutants by a mutagen the occurrence of mutants may exhibit a saturation in which the number of mutants may not be proportional to the dose or concentration of

[1] Received for publication January 31, 1955.
[2] I am grateful to Dr. Henry S. Kaplan for several helpful suggestions.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

mutagen, but may become independent of the concentration at high enough doses (*7–9*). There does not appear to be convincing evidence which indicates whether or not these effects occur during chemical or radiation carcinogenesis.

The treated tissue in which the tumors develop is assumed to consist of N_o cells all assumed equivalent.[4] The carcinogen is assumed to cause one or more relevant changes in one or more cells, which will allow the cells to develop into potential tumor cells. The probability of a change in a cell is assumed to be proportional to the product of the total number of cells treated and the concentration of carcinogen (*2*). If a change is a rare event so that only a small fraction of the treated cells undergo changes, the number of cells in which a relevant change has occurred will be given by

$$N_r = \int N_o(t)\, c(t)\, \lambda\,(t)\, dt$$

where $c(t)$ is the concentration of carcinogen as a function of the time, and λ is a constant of proportionality (which may also depend upon the time), called the carcinogenicity constant by Iversen and Arley (*2*).

Carcinogenicity constant.—The value of this constant is not known. It does not have to be known for dose-response studies, provided that its value is independent of the dose of carcinogen and that it is constant for the duration of the exposure of the cells to the carcinogen. There is reason to believe that λ may change during or after a single exposure to a carcinogen, since the observed occurrence of tumors may depend significantly upon whether a given total dose of carcinogen is administered in a single treatment or divided among two or more treatments (*10, 11*). To avoid complications in interpretation from this source, it would be desirable, for the present analysis, to use a single application of a carcinogen which acts for a short enough time interval so that λ would be a constant during the exposure. Further studies using only two applications of carcinogen and varying the time interval between the two treatments might yield valuable interpretable data.

Concentration of carcinogen.—In general, the total amount of carcinogen given the experimental animal is known but not the concentration of carcinogen at each cell. The concentration of carcinogen does not have to be known, but it is important that the concentration at each of the treated cells be proportional to the dose, D, *i.e.*,

$$c(t) = Df(t)$$

where $f(t)$ is independent of the dose but depends on the size of the animal, its specific metabolic pattern, and possibly the location of the cell in the tissue.

[4] It may be that the cells of a tissue are not equivalent, but can be divided into a small number of susceptible and a large number of resistant cells. If so, when the number of cells which have experienced a change becomes an appreciable fraction of the total number of susceptible cells, a deviation of the linear response to dose in the direction of a saturation would be expected (*2*). This possibility will be ignored in the present analysis.

The probability of a particular relevant change, P_r, under the most simple circumstance, when N_o is a constant during the treatment, is then

$$P_r=\frac{N_r}{N_o}=D\int f(t)\,dt$$

$$=k_rD$$

where N_r is the number of cells in which the relevant change has occurred and k_r is a constant. If n independent changes are required, the probability of producing a potential tumor cell should be proportional to the *nth* power of the dose, *i.e.*, $P=P_1P_2\cdots P_n=k_1k_2\cdots k_nD^n$ or

$$P=\frac{N_p}{N_o}=KD^n$$

where N_p is the number of potential tumor cells, *i.e.*, the number of cells which sustained the n carcinogen-induced changes required to change a normal cell into a potential tumor cell.

In order to estimate N_p, each cell must be allowed to multiply sufficiently to be recognized as giving rise to a tumor. Since it may take different time intervals for tumors to arise from different cells (different latent periods), a valid estimate of the total number of potential tumor cells may be expected by waiting long enough for each of them to yield a tumor.

Correction for lethal effects of carcinogens.—Many carcinogens are toxic and therefore may have two effects: they may transform normal cells into potential tumor cells, and they may kill some of the treated cells. If it is assumed that all treated cells are equally likely to be killed (whether or not they have been transformed into potential tumor cells), the fraction of the cells surviving treatment which give rise to tumors may be expected to be the same as if there were no lethal effects. On the other hand, the number of tumors which appear cannot be expected to be a proper measure of the number of original potential tumor cells, since some of the latter will have been killed. To obtain an estimate of the original number of potential tumor cells, it is necessary to divide the observed number of tumors by the fraction of the treated cells which survive treatment.[5] For the purposes of dose-response analysis, a correction of this type should be made if the fraction of the cells surviving treatment is carcinogen dose-dependent. Of course, it is not certain that potential tumor cells and other cells are equally likely to be killed by the carcinogen. To avoid the need for such a correction, it appears advantageous to study the induction of tumors in a tissue using a carcinogen which does not cause cell death in that tissue.

Further comments.—It must be recognized that if the dose-response data for some tissue-carcinogen pair fit the predictions of the mutation hypothe-

[5] Tissues do not consist of a single cell type. In certain cases it may be that several cell types give rise to the same type of tumor, with each cell type having a different carcinogenicity constant and a different susceptibility to lethal effects of the carcinogen. In the absence of experimental data, it does not appear profitable to take such details into account.

sis and yield a small integral value for the estimated number of changes that must occur to transform a normal cell into a potential tumor cell, this fact cannot be considered proof of the mutation hypothesis. The expression "somatic mutation" is not operationally defined. "Mutation" is properly used in connection with certain changes in gametes. Tests for determining whether a particular observed change is a mutation require a transfer of genetic material from one cell to another. Such tests have not yet been performed for tumor cells and it is therefore not possible to decide whether an observed carcinogenic change is the result of one or more mutations or even whether the change is nuclear. Moreover, if several changes were required, they might not all need to occur in the same cell. If a micro-colony of n similar singly changed cells were required to initiate the growth of a tumor, the same form of dose-response relation would be observed (*12*) as if a single cell required n changes.

Comments on Experiments

Although there are many uncertainties in the interpretation of dose-response data from the standpoint of the mutation hypothesis, it is important to recognize that at the present time progress in this type of analysis is limited not by theory, but largely by the almost complete lack of suitable experimental data. The suitability of most experimental data is impaired by difficulties in making a proper estimate of the number of tumors produced, by complications which are a consequence of the method of administering the carcinogen, and by dose-dependent indirect effects of carcinogens.

Incidence.—According to the mutation hypothesis, a tumor arises from a potential tumor cell, the animal being a complicated growth medium for the cell; the proper way to count these cells is to count the observed number of tumors or its equivalent, the average number of tumors per animal at a time after treatment such that all potential tumor cells will have given rise to tumors. In practice it may be difficult to count all the tumors directly because 1) new tumors may appear over such a wide range of time intervals after treatment that the experimental animals may die from the first tumor before all potential tumors are detected,[6] 2) if the tumor metastasizes, it may be difficult to distinguish between a metastasis and a tumor of independent origin, and 3) the early tumors may coalesce, resulting in an underestimate when scoring late. In some studies there is a significant incidence of spontaneous tumors, and the proper way to correct for these is uncertain. If an attempt is made to score tumors shortly after treatment, there will undoubtedly be errors because of missing micro-tumors. This would not be a serious complication in making estimates of n, the number of changes, if the same fraction of all potential tumors were counted for all doses of carcinogen. However, in certain studies the latent period of the tumors depends upon the dose of

[6] From the standpoint of the mutation hypothesis, an important objection to the analysis by Charles and Luce-Clausen (*1*) is that they did not obtain data on all the tumors which might have appeared as a result of the skin paintings. Part of the variation in the number of tumors that appeared as a function of the duration of treatment is due to latent-period effects.

carcinogen (*13, 14*), so that the fraction of the tumors which will be counted might be expected to vary with the dose of carcinogen. To avoid concern over this effect it is desirable to demonstrate the same tumor incidence in duplicate groups killed at significantly different time intervals after treatment.

Poisson's distribution.—In many experimental studies the fraction of the treated animals yielding tumors (incidence) is reported instead of the average number of tumors per animal. These two methods of reporting data are, in principle, equivalent in the special case in which the tumors are distributed at random among the hosts, *i.e.*, in the case where all hosts are equally susceptible (*15*) and the probability that a host gets a tumor is independent of the presence of other tumors in the host. In this case the relation between these two methods of reporting data is governed by Poisson's distribution:

$$F_x = \frac{e^{-T}T^x}{x!}$$

where F_x=the fraction of the animals having exactly x tumors when T=the average number of tumors per animal. Hence, $F_o = e^{-T}$ is the fraction of the animals having no tumors. The average number of tumors per animal is then

$$T = -\ln F_o \quad , \text{or}$$

$$T = -\ln (1 - \text{incidence}).$$

The problem of determining the average number of tumors per animal would be much simplified if tumors were distributed purely at random according to the Poisson distribution. In this experimental situation it would not be necessary to count tumors, but simply to determine accurately the fraction of the treated animals without tumors.

Tests of whether Poisson's distribution actually describes the distribution of tumors among animals have been reported by Haddow (*16*), Brues (*17*), and by Polissar and Shimkin (*15*). Brues reported that tumors produced in mice by Sr^{89} were distributed in accordance with Poisson's distribution, but he presented no data. The other authors—Haddow (*16*) for spontaneous mammary tumors, and Polissar and Shimkin (*15*) for lung tumors—reported significant deviations from Poisson's distribution which they considered to be a consequence of a nonuniform animal population.

Dose of carcinogen.—It is difficult for a number of reasons to obtain a proper estimate of the dose or the concentration of the carcinogen at the tissue site under study: 1) the carcinogen may exist in different chemical states, such as free or protein-bound; 2) the concentration of the carcinogen may vary with time after administration in a way which depends upon the dose; 3) the incidence of certain tumors depends not only on the total dose, but on whether the total dose is administered in one or in several treatments and on the time interval between treat-

ments (*10, 11*); and 4) the distribution of the carcinogen may be nonuniform in the tissue in which the tumors arise.

The nonuniform distribution of the carcinogen imposes a difficulty in interpreting certain studies, *e.g.*, the incidence of lung tumors induced by intravenous injection of a suspension of an insoluble carcinogen. In this study, tumors appear to be related to the larger granules of carcinogen localized in the lung (*18*). Carcinogen concentration is relatively low and independent of carcinogen dose at cells localized away from the immediate vicinity of granules, and high and independent of the dose at cells near granules. Changing the dose administered might be expected simply to change the number of large granules and thereby the number of sites at which tumors could arise. The observed proportionality between tumor incidence and dose might mean only that the number of tumors is proportional to the number of sites at which large granules are localized.

This difficulty in the interpretation of dose-response data for production of pulmonary tumors by suspensions of insoluble carcinogens appears to be avoided in the studies using urethan, a soluble carcinogen. Unfortunately, in the investigations using urethan, tumors were scored before all could be expected to have appeared. This may produce an error (as discussed above) if, as with many other carcinogens, the latent period varies with the dose of carcinogen. In addition, there is disagreement between the results obtained in the two investigations of the dose-response relation, since Henshaw and Meyer (*19*) found proportionality between the average number of lung tumors per mouse and dose of urethan, whereas Rogers (*10*) found a lack of proportionality.

The studies by Kaplan and Brown (*11*) of thymoma induction by X irradiation of strain C57BL mice appear to avoid most of the experimental difficulties mentioned above. Although the radiation dose is probably not quite uniform in the thymus gland, the dose at any point is proportional to the dose in air. Induced tumors appear within the first year after irradiation, and further thymoma incidence after this time is negligible. It is therefore possible to determine the fraction of the treated animals in which thymomas will not occur. On the other hand, it does not seem possible to measure the average number of tumors per mouse because of early lethality and dissemination; and it has been necessary for the present analysis to estimate this number from the fraction of the mice without thymomas, assuming

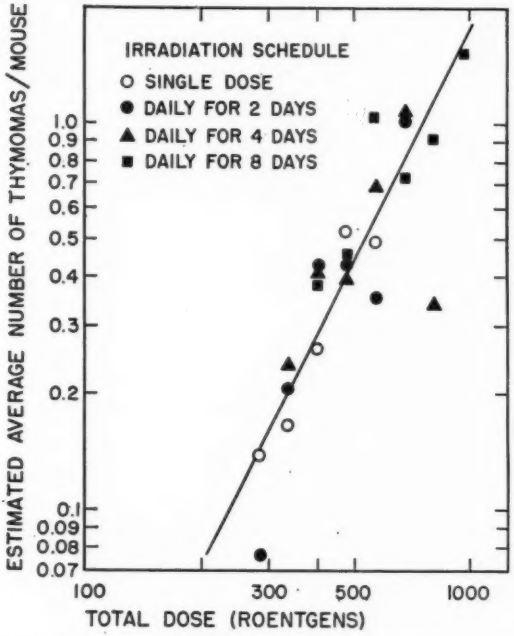
$$N = 1 - n(1 - \text{incidence})$$

as discussed above.[7] The data used in this analysis are for mice in which the total dose of X rays was given in a single treatment or divided into daily treatments for 2, 4, or 8 days. The log of the estimated average number of tumors per mouse *versus* log dose of radiation has a slope of 2 (text-fig. 1), indicating that the average number of tumors per mouse is proportional to the square of the dose of radiation. In order to interpret

[7] Unfortunately, it is not certain that this assumption is valid, and it would be very difficult to demonstrate its validity for X-ray-induced thymomas.

this relation, it would be necessary to correct the observed tumor incidence (as discussed above) for the loss of tumors due to thymolytic effects of X rays.

Indirect effects of carcinogens.—The incidence of certain carcinogen-induced tumors can be modified significantly by underfeeding (*20*), by administration of sex hormones (*21, 22*) or adrenocortical hormones (*21, 22*), by removal of glands such as the pituitary (*22*), and, in the case of X-ray-induced thymomas, by administration of bone marrow (*23*). These facts cannot in themselves be considered as evidence against the mutation hypothesis. However, food intake, endogenous secretion of hormones, and, in the case of X rays, the bone marrow may be modified by the administration of certain carcinogens to an extent which depends upon the dose. The observed incidence of carcinogen-induced tumors may therefore be the resultant of two types of effects of carcinogens, direct effects and indirect effects, both of which may depend upon the dose. Since mutation production might be expected to depend upon the direct effect of the carcinogen on the cell, it appears to be important to correct the observed dose-response curves for the effect of dose-dependent indirect effects. Only then can one expect estimation of the number of carcinogen-induced changes required for the transition to potential



TEXT-FIGURE 1.—The relation between the estimated average number of thymomas per mouse and the total dose of X rays. The average number of thymomas per mouse was estimated from the fraction of the mice without thymomas, assuming that thymomas were distributed among treated mice according to the Poisson distribution.

tumor cell to be possible. Since the contribution of the effect of the X irradiation of the bone marrow to thymoma induction is not known as a function of dose, it is not yet possible to evaluate the significance of the observation that the average number of tumors is proportional to the square of the dose, *i.e.*, to estimate the number of radiation-induced changes (if any) required for the transition from thymus cell to thymoma.

Summary of criteria for dose-response data suitable for analysis from standpoint of mutation hypothesis.—The tissue should be relatively stable, not appreciably affected by hormonal level and other influences which might be changed by carcinogen administration. The carcinogen should be rapidly metabolized or excreted or have action of short duration. It should induce tumors after only a single dose. The concentration of carcinogen at each cell should be proportional to dose. The carcinogen should not kill cells in the tissue to any appreciable extent. It is desirable to be able to count individual tumors induced by the carcinogen and to demonstrate a plateauing of the incidence of tumors. It is also desirable to be able to demonstrate lack of, or to be able to correct for, dose-dependent indirect effects.

Data from experimental situations which deviate from these criteria may still be very valuable for the type of analysis discussed, but supplementary data will be necessary to make required corrections or to check assumptions.

Age-incidence analyses.—Several analyses have recently appeared that attempt to estimate the number of mutations required for the spontaneous transition of normal tissue to tumor from age-incidence data (*10*, *24*, *25*). However, peculiarities in the age-incidence data for spontaneous tumors of various organs (*26*) suggest that age-dependent naturally occurring carcinogenic activity or that age-dependent modifying influences may be operating. In addition, the latent period between the carcinogenic change in a cell and the observation of the tumor is, in general, of unknown duration at any age. In the absence of data on the magnitude of such effects, it is desirable to have reservations on the quantitative significance of such analyses.

Mechanisms of modification of tumor incidence.—The mechanisms by which various treatments modify tumor incidence are unknown. From the standpoint of the mutation hypothesis the number of tumors would be expected to vary directly with the number of cells surviving treatment. It is of interest that Kaplan (*21*) has already noted the parallel between the action of treatments that change the thymus weight and the incidence of X-ray-induced thymomas. On the basis of this hypothesis, the exceptional action of estrogen in causing an increase in thymoma incidence despite its inducing thymic involution would be interpreted not as being a contradiction to the hypothesis, but as indicating that estrogen has another effect in addition to its effect on thymic weight. For an estimate of the magnitude of this additional effect, a correction would have to be made for the decrease in the number of potential tumor cells associated with the decrease in thymic weight.

Relative importance of direct and indirect effects.—The relative importance of direct and indirect effects in determining differences in incidence of carcinogen-induced tumors in various strains of animals is not yet generally established. In a few studies on incidence of carcinogen-induced lung tumors, however, differences in susceptibility between strains resided in the tissue and not in indirect effects of the host on the tissue (*27*, *28*). When the difference between the strains resides in the tissue, it would be of interest to know whether it is due to a difference in the apparent number of changes required. In the case of X-ray-induced thymomas, the indirect effect of the radiation appears to be very large. There is no evidence as yet that a direct effect of radiation on the thymus plays a part in the induction of thymomas. On the other hand, it cannot yet be concluded that a direct effect does not occur.

Summary

A theoretical analysis is made of the dependence of tumor incidence on dose of carcinogen that might be expected from the standpoint of the mutation hypothesis. Tests of this analysis do not appear possible at the present time because of the absence of suitable experimental data. The suitability of most data is impaired by complications which are a consequence of the methods of administering carcinogens, by difficulties in making estimates of the number of tumors produced, and by dose-dependent lethal effects and indirect effects of the carcinogens.

The induction of lung tumors by chemical carcinogens, and the induction of thymomas by X rays were discussed as examples.

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The Role of Hair Follicles in Methylcholanthrene Carcinogenesis in Mice[1,2]

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Increasing attention is being paid to the role of hair follicles in the sequences of experimental carcinogenesis (*1–4*). Some tumors are believed to originate in the hair follicles, but direct visible evidence of papillomas and carcinomas actually arising from them has not been established. Only the early changes of hair follicles, in response to three paintings with 0.6-percent methylcholanthrene in benzene applied from 7 to 30 days after the initial application, have been studied in epidermal whole mounts in which the attached hair follicles are separated from the underlying dermis by maceration in cold dilute acetic acid (*5*). Atrophied, enlarged, and fused hair follicles were observed. These findings were confirmed by the examination of stained sections of skin that had been subjected to a single application of the same carcinogen and observed for a period of 15 days (*6*). In addition to the three kinds of altered hair follicles observed in the whole mounts, hair follicles changed in another way were seen. Since these follicles had lost their connections with the epidermis, it is not surprising that they were not seen in the whole mounts of epidermis.

It can be proved by complete serial sections that some of these masses of epithelial cells are not connected with epidermis. Since we cannot be sure that all of these masses of cells develop from hair follicles, the possibility remains that some may be of epidermal origin—though this does not seem likely. It is possible in whole mounts to establish a gradation in properties between selected follicles fixed in the process of enlargement and cysts; but, as in all studies of histogenesis, the same follicle cannot be seen to undergo this change because its development is cut short by killing the tissue in making the preparation.

Some follicles were transformed into clublike, solid clusters of cells devoid of hairs and sebaceous glands. From them cystic formations developed, either connected with hyperplastic epidermis or completely

[1] Received for publication April 26, 1954.
[2] Aided by grants from the Charles F. Kettering Foundation and the American Cancer Society.
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enclosed by hyperplastic epithelial walls lying in the dermis. Cytologically, the changes that occurred in the hair follicles resembled those in the epidermis. But their role in chemical carcinogenesis remained to be determined. The purpose of the present study is to determine whether the altered hair follicles, observed during the early stages of carcinogenesis, persist and give rise to papillomas and carcinomas. This study is based mainly on the examination of epidermal whole mounts supplemented by microscopic sections.

Material and Methods

Fifty 5-week-old inbred female mice of the Swiss strain were used. They were kept on Rockland mouse diet. In order to make our observations directly comparable with those of earlier workers in this laboratory, who paid no special attention to the state of the hair cycles, the same standard technique of painting was followed (*7*). Animals 5 weeks of age were chosen because in mice at that age 2 generations of hair cycles have usually been completed (*8*). Generally, there is less variability in the stages of the follicular cycle of hair in young mice of the same age and sex than there is in older mice (*8*, *9*).

The backs of the animals were painted with 0.6-percent methylcholanthrene in pure benzene 3 times a week for 4 weeks. A No. 4 camel's-hair brush was used. The painted areas were examined once every other day for 100 days with the aid of a 6× magnifying glass. Special attention was paid to alterations that seemed to be progressive.

Mice showing similar lesions were selected, separated into groups and then sacrificed at 5-day intervals. In one group, the skins of the painted areas were excised and the epidermis was separated from the dermis with cold acetic acid—exactly as already described (*5*). Both stained and unstained epidermal whole mounts were made. The whole-mount preparations were studied under a stereoscopic binocular microscope with 3× objective and 9× ocular, in order to get a three-dimensional view of the altered follicles.

From the other group of animals (which showed lesions corresponding to those in the whole mounts) the skins were excised and, after being carefully spread and oriented on a piece of filter paper, were fixed in Bouin's fluid. Serial paraffin axial sections of these skins (10 microns in thickness) were stained with modified Masson's trichrome (*10*).

Interesting observations of the epidermal whole mounts were confirmed by the study of these microscopic sections. Both types of altered hair follicles (connected with and disconnected from the epidermis) definitely developed into papillomatous formations and carcinomas. However, their modes of growth during the course of carcinogenesis were different. In one type they developed into papillomas and stayed benign for a long period; in the other, signs of malignancy appeared much earlier. Individual variations were observed among different mice as well as localized variations between different parts of the treated areas.

Observations

Tumors from the Altered Follicles Connected with the Epidermis

Rudiments of the prospective tumors derived from such altered follicles, and as yet invisible to the naked eye, were observed in the epidermal whole mounts as groups of enlarged or fused follicles as early as 10 days after the initial painting (fig. 5). These rudiments later developed into typical broad-based or narrow-based papillomas when the involved follicles were closely packed together, or they developed into elevated ridges and granulations with no definite shape when the involved follicles were more widely scattered. Early signs of such papillomatous formations were first detected in the painted area with the aid of a 6× magnifying glass, about 20 days after the first painting.

1) *Typical papillomas.*—Broad-based and narrow-based papillomas were observed. They were both formed by the enlarged and fused follicles gathered into rounded masses. Figure 1 is an unstained preparation of an epidermal whole mount viewed from the outer surface of the skin, 40 days after the first painting. It shows four broad-based papillomas of different sizes lying in a row. The two papillomas in the center were purposely pushed down a little. Figure 2 is the same specimen turned over to show the other (inner) side. It is obvious that the bulbs of these papillomas were formed by groups of enlarged and fused hair follicles (*cf.* fig. 6 viewed from the inner side). In figure 2 the papilloma on the right is seen to be made up of a group of more distinct and greatly enlarged follicles. Whereas, in the one on the left the individualities of the altered follicles are not so clearly defined; they are, however, likewise formed by the fused follicles.

Microscopic sections, showing corresponding changes, are illustrated in figures 3 and 4. Figure 3 shows a section of a papilloma formed by the fused hair follicles. These follicles have in some cases penetrated into the dermis and subcutis and look almost malignant, in that the growth appears to be somewhat disorderly with the cells small and lacking in reactions to stains. The papilloma illustrated in figure 4 is formed mainly by the enlarged hair follicles, and one notices layers of dense-looking, thickened dermal tissues forming a sort of capsule around them. These capsules are better seen in the dermal whole mounts (as shown in fig. 8) which will be referred to later.

The possibility that cells of hair-follicular origin are caught secondarily in papillomas of epidermal origin is a remote one and we do not advance it.

Typical broad-based papillomas are round or oval in shape. Such a papilloma usually consists of greatly enlarged or fused follicles at its center and elongated hypertrophic or normal-looking follicles at its periphery (fig. 6). The latter act like nails and anchor the bulk of the papilloma into the dermis (fig. 4), thereby making it firm and immovable when touched and placing it in the above classification.

Narrow-based papillomas are more elevated above the surface—possessing short, narrow, pediclelike bases—and dangle rather loosely when

touched. They are also formed by altered follicles connecting with the epidermis; but they are pushed outward toward the surface instead of being sunk deeply in the dermis. Figure 14 shows such a papilloma in an epidermal whole mount, viewed from the inner, dermal side. Note that there is a hollow space, which originally contained dermis, inside the papilloma; that the base is bounded by a constricted rim of epidermis; and that anchoring nail-like follicles are lacking. The papilloma is somewhat pinched off from the dermis. Narrow-based papillomas usually grow slowly and become benign horny warts, but sometimes they wither and disappear.

2) *Atypical papillomas.*—If the early responding follicles are somewhat scattered in any particular area, instead of being closely packed together, their future behavior will be different. Papillomas derived from scattered follicles appeared later in the skin—either as elongated, elevated ridges or as a series of aggregated granulations with no definite shape—even though they are composed of the same type of altered follicles as are typical papillomas. Like the typical broad-based papilloma, when felt, they seem to be firm and immovable. Figure 7 is from a whole-mount specimen, 55 days after the first painting. It shows such a papillomatous formation viewed from its inner side. Compare this formation with figure 6 and note that both are formed by the same type of altered follicles.

In both the papillomas and the elevated granulations, the individual constituent follicles are encapsulated by the dermal tissues. This encapsulation is shown very clearly in figure 8 and in the corresponding dermal whole mount of figure 7, which is the dermal bed from which the epithelial papillomas represented in figure 8 have been removed. Observe in it the capsular compartments which lodge the individual enlarged follicles. The capsules appear in the supplementing microscopic sections as layers of dense dermal tissue (fig. 4).

These encapsulated papillomatous formations usually grow slowly and stay benign for a long time. They finally become malignant, if preceded by the ulceration that occurs at approximately 50 to 80 days after the first painting. In our series, all the ulcerated papillomas examined in stained sections were malignant and contained some cells of epithelial origin in the process of invading neighboring tissues. Grossly or microscopically demonstrable ulcerations in such papillomas strongly suggest malignancy.

Tumors from Altered Follicles Disconnected from the Epidermis

The papillomatous formations described thus far are derived from modified hair follicles connected with the epidermis. Hair follicles that have lost their continuity with the epidermis and consist of isolated masses of cells lying in the dermis, also participate in the formation of papillomas. Papillomas derived from them generally appear to differ from the other types. They usually become malignant much earlier without becoming ulcerated.

In two instances, definite neoplastic changes were observed within such isolated masses of follicular cells. First, figure 9 shows a mouse, 40 days after the first painting, with interscapular skin distended by a protruding ulcerating structure. On the 20th day after painting, this structure felt like a hard mass in the dermis and increased rapidly in size, while the surface skin became greatly distended. Sections cut through the center confirmed the observations that this growing mass originated from epithelium in the dermis because the surface skin showed little connection with the underlying tissue (fig. 10) and that it was already an advanced carcinoma (fig. 11). The other lesions in the lumbar region of this animal (fig. 9) are clearly of a different nature.

Second, figure 12 is the other case, 50 days after painting, in which the invading cells did not occupy the whole dermal tissue and therefore they could be seen spreading from an isolated clump of follicular cells (fig. 13). Since the early stages were not observed, it is possible that the malignant cells may have been of epidermal rather than of follicular origin. In both cases, dermal capsules surrounding the tumors were not observed.

Modes of Growth

Our observations revealed that at least two modes of growth are exhibited by the altered hair follicles during the course of carcinogenesis.

1) *Cumulative growth* is adopted by the altered follicles that are connected to the epidermis. This type of growth is characterized by a local increase in size, transformation into a cyst, and encapsulation by the dermis (figs. 4 and 8). Groups of such cysts form the main bulbs of the papillomatous formations. They stay benign for a longer period, and become malignant later if the capsules are destroyed by ulceration.

2) *Diffusive growth* is adopted by the altered follicles that have lost their continuities with the epidermis and have become groups of isolated masses of follicular cells scattered in the dermis. This growth is characterized by invasion of the dermis and absence of protective dermal capsules, and it also gives rise to malignant cells much earlier whether ulceration occurs or not.

Atrophic Follicles and Healed Ulcers

Atrophic follicles (that is, smaller than usual) observed in the two previous studies (*5*, *6*) were also found in this series. In some animals, the painted area became epilated. The hairs were shed at 6 to 9 days after painting. The bald area turned yellowish in color and ulcerations usually occurred in about 10 to 14 days. Hair follicles were either severely damaged or completely destroyed. The ulcers healed approximately 4 weeks after the first painting. Healing of the ulcers proceeded from the periphery toward the center. The healed ulcers usually appeared as shallow depressions with fairly smooth shiny surfaces and slightly elevated margins. They remained bare for at least 2 or more weeks, or even permanently. Regeneration of hairs ordinarily started

from the margins of the healed areas. Papillomatous growths in these animals were most frequently found at the margins of the healed areas and in the ulcerated area which is the last to heal.

A three-dimensional view of such an area is instructive. Figure 16 shows a whole-mount preparation of a healed ulcer, viewed from its dermal side and stained with Harris' hematoxylin. Unlike its outer surface, which is smooth and shiny, the inner surface is roughened by the presence of many epithelial foldings which interlock with the underlying dermis. On the left, and along its upper margin, one can still recognize traces of the altered follicles as darkly stained protrusions, likely to develop later into papillomas. At its center, the roughened surface is formed by the merging of the damaged abnormal hair follicles and the regenerated epidermis which becomes much thickened and sinks into the dermis. The clear spaces shown in this photograph were occupied by the dermal tissues in the intact animal.

Figure 17 also shows an epidermal whole mount, stained with hematoxylin but which had separated during the active phase of the hair cycle. The long-growing normal follicles can be recognized as darkly stained stripes in both the upper and the lower third of this picture. The middle third of this figure is the healed ulcer. In this case, the merging of the damaged hair follicles and the epidermis during their course of reparation following ulceration is so advanced that traces of altered follicles can hardly be identified. The healed area, under such conditions, appears somewhat like a honeycomb, in which the intensely black, sharply outlined areas of quite variable size, often limited by clear areas, are the epithelial cells, and the clear spaces are filled by the dermal tissues. A healed ulcer of this type usually remains bare during the life of the animal. A section across it would show the epithelial cells in isolated islets, or cords, intermingling with the dermal tissues and would give the impression of an invasive growth.

Types of Follicles and Their Response

In our first contribution (*6*), the principal arrangements of hair follicles on the backs of the mice and the two main types of follicles (sensory and ordinary) were described and illustrated in a dermal whole mount. The basic grouping of the follicles is a pattern of trios. Scattered among the trios are solitary follicles, which are the largest in size and are more richly supplied with nerves so that they can be classified as sensory.

Ontogenetic studies of hair-follicular arrangement in the mouse by various workers have been reviewed by Noback (*11*). According to Noback, the first follicles to differentiate are the central trio follicles. The trio is formed when two follicles are differentiated lateral to the primary follicles. If no lateral follicles differentiate, the primary follicle becomes a solitary follicle. Follicles of the next generation (secondary follicles) are located between the central trio follicles and the lateral trio follicles.

In regard to the types of hairs produced by these follicles, it can be said that, in general, the central trio follicles form the coarse overhair, while the lateral trio follicles form the fine underfur (*12*). In our series, the solitary sensory follicles form the coarsest and longest hairs. Their normal behavior in the process of keratinization may be different from that of the lateral trio follicles.

Therefore, though the animals are painted at the same stage of their hair cycle, the initial properties of the hair follicles, in respect to sequence of differentiation (age), richness of nerve supply, and types of hairs formed, are not the same even in the same animal. A different response by the various follicles to the same carcinogen is to be expected.

Unfortunately, the characteristic pattern and the morphology of the individual follicles are so greatly modified after painting that it is almost impossible to trace their individual identities. However, in the less affected areas, dermal whole mounts, specially stained to show the nerves (*13*), indicate that the sensory follicles were more severely affected.

Figure 18 illustrates the specially stained dermal whole mount, 20 days after painting. It shows a much enlarged solitary sensory follicle among many less affected ordinary trios. Note the tortuous nerve networks and the clawlike endings supplying this sensory follicle. Compare the papillomas of figure 18 with the papillomas illustrated in figures 2, 6, and 7, in which one finds in the bulbs of the papillomas one or two altered follicles of much larger size. But whether these larger altered follicles are derived from the solitary sensory follicles remains unknown because it is not possible to observe the behavior of individual follicles over long periods *in vivo*.

Papilloma Size, Growth Rate, and Cyclic Changes in the Skin

The size and the growth rate of the papillomas vary under different conditions. When two papillomas occur at the same time, one factor that determines their size is the number of the altered follicles involved. The more follicles there are, the greater their size is likely to be, as shown in figure 6, in which the papilloma represented in the upper left corner consists of only a few altered follicles. However, one of the altered follicles in this papilloma is two or three times larger in size than those in the papilloma on the right. But the size of the papilloma on the lower left as a whole is only about one third the size of the other.

Papilloma size may also fluctuate with periodic local changes of the skin. In the normal skin of the mouse, there is a cyclic change of large magnitude in the thickness of the epidermis, the dermis and especially of the subcutis, as has been described by us (*6*) and by others (*8, 14*). After painting, the hair follicles are altered by the carcinogen but the periodic changes in volume of the skin persist in spite of repeated painting with methylcholanthrene. Evidently when the local underlying dermis and subcutis of a papilloma become thickened, the papilloma appears to be larger and more prominent than when the skin is thinner.

The growth rate of the papillomas varies with their types. In general, papillomas which undergo "diffusive growth" grow much faster than papillomas which enlarge by "cumulative growth."

The tumor in the interscapular region illustrated in figure 9 shows the diffusive type of growth. As already stated, this particular tumor increased considerably in size within 20 days. While in cumulative growth, the broad-based papillomas grow faster than the narrow-based ones. But the growth rate is also affected by the general condition and the constitution of the animals. Papillomas in weaker animals usually grow more slowly than those in the vigorous animals.

Figure 15 illustrates a retarded papilloma of a weak and thin-skinned animal, 50 days after the first painting. The size of this papilloma remained the same for 30 days. Apparently nutrition is also an important factor in conditioning the growth rate of a papilloma.

Discussion

Origin of tumor.—The evidence presented shows that papillomatous formations are derived from follicles severely altered in consequence of applications of methylcholanthrene and that carcinomas often develop in them. A special search was not made for carcinomas arising in epidermis.

Papillomas, which arose from hair follicles after tar painting, were mentioned by Guldberg (*15*). Using methylcholanthrene, Orr (*16*) found that only a proportion of the tumors developing from the walls of cysts were derived from the hair follicles.

Earlier workers usually examined their specimens in stained sections only. When only a few sections in a series were studied it is possible, even likely, that the information obtained concerning whole follicles and sebaceous glands was inadequate. Both hairs and sebaceous glands were destroyed by the carcinogen (*17*, *18*). Perhaps they interpreted altered follicles (deprived of hairs and sebaceous glands) as epidermal downgrowths and emphasized too greatly the epidermal origin of carcinomas. Employing benzpyrene, Glücksmann (*19*) recognized the occurrence of a malignant change in hyperplastic hair follicles as we have done.

Failure to induce tumors in the epidermis of the soles of the feet (where there are no hair follicles) of mice which ran daily on plates smeared with the carcinogen, whale oil (*20*), is significant. Moreover, when the hair follicles in the skin are destroyed by ultraviolet irradiation, the skin becomes resistant to the carcinogenetic action of methylcholanthrene, but the same carcinogen will induce rapidly growing cancers when applied to skins in which the hair follicles have regenerated (*21*).

The extent of exposure of susceptible cells to carcinogens is a factor to be considered in any effort to assess the likelihood that these cells will undergo malignant transformations. The great thickness of epidermis covering the soles of the feet of these mice, the probable increase in thickness of epidermis resulting from ultraviolet irradiation, and the absence of hair follicles may all be linked with the failure of development

of cancer in response to methylcholanthrene. That the follicles are portals of entry for methylcholanthrene in benzene has been demonstrated (*17*). Modifications in the epidermal cells have been discussed by many investigators (*9*, *22*, *23*).

More recently, the importance of the hair-follicle cycle in experimental skin carcinogenesis has been emphasized in mice (*1*, *4*) and in rabbits (*3*). In both cases, tumors are believed to originate in hair follicles.

In sheets of epidermis in whole mounts after painting with methylcholanthrene, Liang (*13*) has found that the first indication of local epidermal change was the appearance of cell clusters in a radiating pattern, usually at the junction of the hair follicles and the basal epidermal layer. Extensive multiplication of these cells caused disruption of the follicular pattern and eventual ulceration in the center of the proliferation area. In stained sections, Cramer and Stowell (*23*) have observed that epithelial cells of the altered hair follicles in the peripheral parts of the ulcers take a prominent part in repair.

We have demonstrated in this study the merging of cells of the altered follicles with those of the epidermis in whole mounts of two healed ulcers—as illustrated in figures 16 and 17. In such a regenerated epidermis the cancers that appear to originate in epidermis do so in epidermis of follicular origin. The reverse never happens, *e.g.*, follicular epithelium being developed from epidermis in adult mice (*4*).

By fluorescence microscopy it has been discovered that after benzpyrene has been painted on mouse skin it becomes localized in the attached hair follicles (*24*). Using the same method, Simpson and Cramer also found that 20-methylcholanthrene is transported through keratin and sebaceous glands into the hair follicles (*17*, *18*). The initial kick delivered thereby, mainly to the follicular epithelium, should not be ignored.

Focal response and individual variations.—It is well known that induced cancers appear within fairly sharply defined foci (*20*). In our two previous experiments (*5*, *6*) as well as in the present one, focal changes of the hair follicles were observed at varying sites in the painted area. Severely modified hair follicles, but not epidermis, later developed into papillomatous growths and carcinomas. Apparently, hair follicles are the primary site of the neoplastic response. Their cells in the late catagen or telogen phase are more susceptible to the carcinogen, whereas the cells of follicles in the midanagen phase are more resistant.

In animals with known phases of hair cycles at the time of painting, Chase and Montagna (*2*) observed a progressive keratosis, epilation, and inhibition of hair growth in areas of the telogen phase, while in areas of the anagen phase, only a moderate keratosis with no inhibition of hair growth was found. It is also interesting that in animals whose hair cycle was checked by biopsy, Andreasen and Engelbreth-Holm (*1*) found a significantly higher incidence of papillomas in animals during the telogen than the anagen phase after a single painting of 9,10-dimethyl-1,2-benzanthracene.

But on the contrary, Wolbach (*4*) pointed out that active hair follicles, with open hair canals, are more vulnerable to the carcinogen than are the quiescent ones with plugged canals. In rabbits painted with 9,10-dimethyl-1,2-benzanthracene in lanolin, Whitely and Ghadially (*3*) also claimed that papillomas appeared at the site of an originally active zone.

Apparently carcinogenic action in the skin depends to some extent on the state of the hair follicular cycles at the time of painting. In our previous study (*6*) we noted great individual differences in phases of the hair-growth cycle in the interscapular region of 15 normal controls of the same strain, age, and sex. In addition, the time of onset of the hair cycles may also be quite different in animals within the same litter (*8*). Even in the same animal, the natural growth of hair does not start simultaneously all over the body but in a wavelike pattern with some regions being in advance of others (*4*, *12*, *25*, *26*). For this reason it is to be expected that local differences in the susceptibility of follicular cells will be encountered in the interscapular area to which the carcinogen is applied in our experiments.

Locally marked increases in subcutaneous fat, blood supply (*26*, *27*), and tissue fluid (*28*) in the active zone may perhaps promote or inhibit the carcinogenetic action of methylcholanthrene. Likewise, the fluctuations in amount of glycogen, acid polysaccharides, alkaline-phosphatase activity, sulfhydryl groups, ribonucleic acid and deoxyribonucleic acid during active and resting periods of the hair-growth cycle (*27*, *29*, *30*) could possibly play controlling roles in the focal response of the hair follicles toward the carcinogen.

Factors controlling hair-follicular activity.—Butcher (*31*) noted that in the prepubertal rat the growth activity of the ovary parallels the hair cycles. But in ovariectomized animals the growth activity of hair germs is not affected. In mice, Bullough (*32*) found that the mitotic frequency of epidermal cells and the thickness of the epidermis, dermis, and subcutis undergo cyclic changes coincident with the phases of the estrous cycle. Resolution of the factors involved is difficult because so many are directly or indirectly cancerous. Thus the activity of the hair germs may be accelerated by administration of thyroxine and adrenalectomy (*33–36*) and inhibited by direct application of adrenal-cortical preparations (*37*), by administration of high doses of estrogen (*38–41*), by thyroidectomy (*42–45*), and by hypophysectomy (*44*, *46*, *47*).

The correspondence in time between increased vascular supply in the dermis and the subcutis has been alluded to. Haddow and Rudall (*25*) attempted to correlate these periodic alterations in capillary activities with rhythmic control exercised by the sympathetic nervous system. However, Durward and Rudall (*48*) later discovered that denervation of an area of the skin failed to affect the normal growth waves of the hair. Hardy (*27*) has recently found that hair follicles in mice were richly vascularized only in the phase of maximum hair growth.

Fluctuation of papilloma size and hair-growth cycle.—This fluctuation was first reported by Guldberg (*15*) in mice treated with tar. He found

that papillomas developed from hair follicles show a periodic hypertrophy and atrophy of growth parallel with the hair-growth cycle. Similar changes of growth in epidermal warts in mice were later observed by Mottram (*49*). Both authors correlated the disappearance and the reappearance of papillomas to periodic change in the skin. More recently, Wolbach (*4*) observed that papilloma cells situated in active hair-growing zones are larger, exhibit more mitotic figures and are more deeply stained by dyes than are those of a papilloma in a quiescent region. In our series, only narrow-based papillomas sometimes disappeared, but the broad-based papillomas also fluctuated in size in accordance with phases of the hair cycle. Therefore, periodic hair-follicular activity persists after repeated application of methylcholanthrene, and may play an important role in the later stages of carcinogenesis as well as in the early stages.

Modes of growth and malignancy.—Malignancy is often assessed by the morphologic features of cellular growth. Consequently, the cumulative and the diffusive modes of growth characteristic of the two different types of altered follicles are of interest. It is possible that diffusive growth is what takes place in the altered follicles which are painted during the active phase of their cycle. In active phases, the growing hair bulbs lie deeply in the subcutis. While the upper (distal) ends of the greatly elongated follicles are destroyed by the carcinogen, the actively growing bulbs are left behind in the dermis. Like regenerating nerve fibers without guiding sheathes of Schwann, their normal polarity of growth is lost.

The neoplastic alterations reported in this study developed in less time than that reported for similar alterations by other workers in this laboratory. In one case (see figs. 10 and 11) an advanced squamous-cell carcinoma was observed on the 40th day after the first painting, although it must have started considerably earlier. Cowdry and Suntzeff (*50*) observed that squamous-cell carcinomas develop more quickly and in a higher percentage of young mice, 2½ to 3 months old, than of 12- to 13-month-old mice. Consequently, the still younger age of 5 weeks, at which we first applied the same carcinogen, may explain why cancers in our experiments appeared so promptly.

Summary

The development of papillomas and carcinomas was studied in the interscapular skin of 50 female mice of the Swiss strain, following cutaneous applications of 0.6-percent methylcholanthrene in benzene 3 times weekly for 4 weeks. The painted areas were examined every second day for 100 days after the first painting of 5-week-old animals, except for those sacrificed for microscopic study of skin specimens at 5-day intervals. Principal reliance was placed on the examination of whole mounts of epithelial and dermal components supplemented by study of stained vertical axial (sagittal) sections of the treated areas.

In addition to epidermal hyperplasia, significant alterations in some hair follicles were observed. These hair follicles either retained connection with the epidermis, or, as scattered masses of cells, lodged in the dermis.

In both cases they gave rise to papillomas, some of which later developed into cancers.

"Cumulative growth" of altered follicles connected with epidermis was characterized by increase in size and encapsulation. Such follicles remained benign for a long period and became malignant later if the capsules were destroyed by ulceration.

Diffusive growth of altered follicles disconnected with epidermis was characterized by absence of protective capsules, invasion of dermis and much earlier formation of cancers whether or not ulceration occurred.

Localized differences in phases of follicular cycle were partly responsible for local and individual responses to the carcinogen. These phases persisted even in the later stages of carcinogenesis.

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PLATE 13

FIGURE 1.—Unstained epidermal whole mount viewed from the outer surface, 40 days after the first painting, showing four broad-based papillomas—the two in the center purposely pushed down a little. × 4

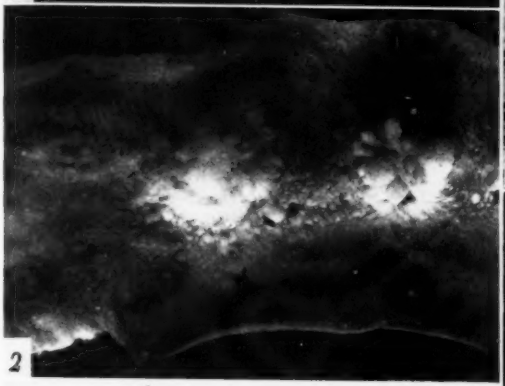
FIGURE 2.—The same specimen as figure 1 viewed from the inner side to show that the bulbs of the papillomas are formed by groups of altered follicles. Note that one of them is formed by enlarged follicles and the other by fused follicles. × 4

FIGURE 3.—Section of a papilloma formed by the fused hair follicles, 40 days after the first painting. Note that the cells have infiltrated to the subcutis. × 16

FIGURE 4.—Section of a papilloma formed by the enlarged hair follicles, 40 days after the first painting. Note the hypertrophied nail-like follicles at its periphery, anchoring the papilloma into the dermis. × 16

FIGURE 5.—Stained epidermal whole mount, 10 days after the first painting, showing that the rudiment of the papilloma is formed by a group of darkly stained altered follicles. × 16

FIGURE 6.—Unstained epidermal whole mount, 50 days after the first painting, viewed from the inner side. A typical broad-based papilloma at the *right*. × 16



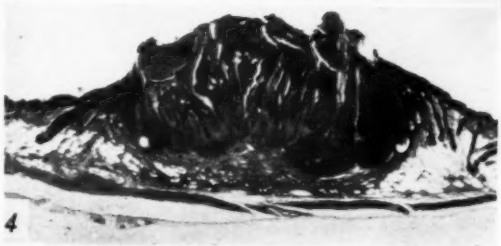


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PLATE 14

FIGURE 7.—Unstained epidermal whole mount, 55 days after the first painting, viewed from the inner (dermal) side, demonstrating that the V-shaped elevated granulation is also formed by the same components as a typical broad-based papilloma. × 16

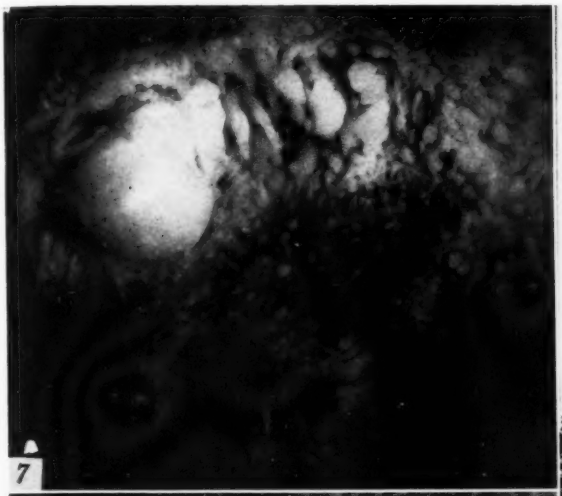
FIGURE 8.—The corresponding dermis of figure 7, viewed from its external (epidermal) side, showing dermal capsular compartments for each group of enlarged follicles. × 16

FIGURE 9.—Two types of papillomatous formations on mouse, 40 days after the first painting. The one in the interscapular region is formed mainly by the altered follicles disconnected from the epidermis. × 1

FIGURE 10.—Section cut through the center of the papilloma in the interscapular region shown in figure 9, demonstrating that the epidermis has but little connection with the underlying mass. × 8

FIGURE 11.—Enlarged portion of figure 10. The growing mass is already an advanced squamous carcinoma. × 85

FIGURE 12.—Sections of two papillomas, 50 days after the first painting. Note the isolated masses of follicular cells in the dermis of the *lower* one. × 8.



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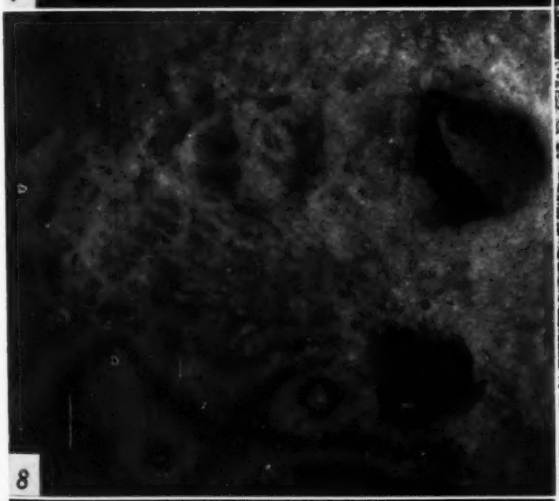

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PLATE 15

FIGURE 13.—Enlarged portion of figure 12 showing the diffusive mode of growth. Note the streams of cells spreading from the isolated masses of follicular cells. × 85

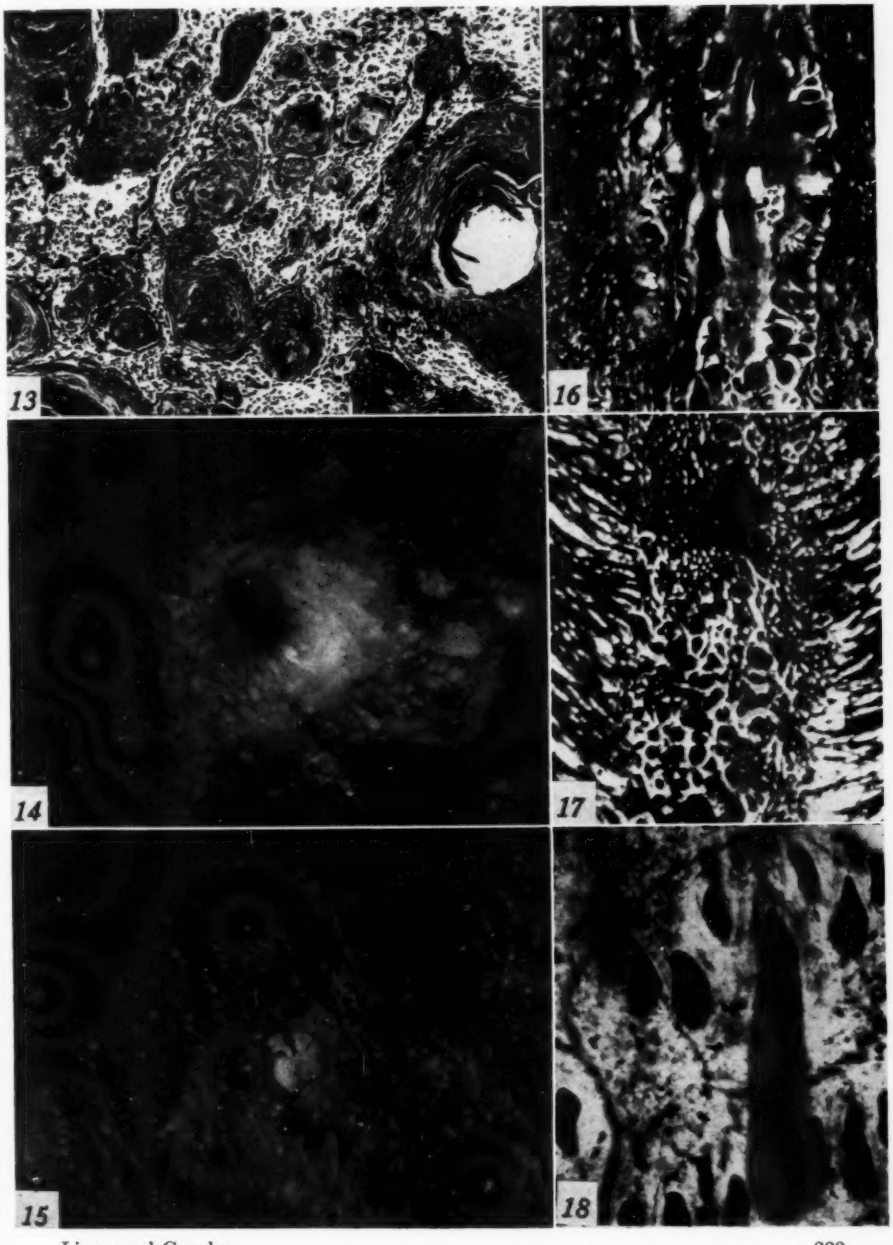
FIGURE 14.—Unstained epidermal whole mounts, 60 days after the first painting, viewed from the dermal side, showing a narrow-based papilloma. Note the constricted rim of epidermis surrounding its base. × 16

FIGURE 15.—Unstained epidermal whole mount, 50 days after the first painting, showing a retarded papilloma. × 16

FIGURE 16.—Stained epidermal whole mount, 45 days after the first painting. An area of healed ulcer in which the clear spaces are the depressions; the elevated parts are darkly stained. Note that its surface is roughened by the presence of many foldings and also the traces of altered follicles appearing as darkly stained protrusions along its upper margin. × 16

FIGURE 17.—Stained epidermal whole mount, 55 days after the first painting, showing an area of healed ulcer devoid of hair follicles. Note the honeycomb-like appearance. × 16

FIGURE 18.—Dermal whole mount (the epidermis and subcutis separated from each other), 20 days after the first painting. The more severely affected sensory hair follicle is surrounded by the ordinary follicles. Note also the tortuous nerve fibers and the clawlike endings supplying the sensory follicle. Schiff's reagent and methyl green. × 16



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The Role of Bile-Acid Derivatives in the Seroflocculation Reaction. I. Preparation of the Bile-Acid Derivatives [1, 2, 3]

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A previous communication (*1*) presented data compatible with the hypothesis of an endogenous carcinogen possessing antigenic properties. This was manifested by the flocculation reaction occurring when the sera of cancer-bearing individuals were mixed with the nonsaponifiable liver fraction ("antigen" A)[5] obtained from patients who had succumbed to cancer. The same reaction was also noted when this fraction was mixed with sera from patients afflicted with other pathologic conditions, but seldom occurred when mixed with the sera of healthy individuals. The carcinogenicity of the nonsaponifiable fraction obtained from cancerous and even noncancerous tissues has been demonstrated by a number of investigators (*2–13*). The antigenicity of a similar fraction has been demonstrated in our own laboratory. In a series of Schultz-Dale reactions, positive responses were obtained from the guts of guinea pigs sensitized with "antigen" A that was mixed with horse or swine serum as "Schlepper." In addition, the injection of "antigen" A plus protein material into rabbits, resulted in a flocculation reaction when the sera of the sensitized animals were mixed individually with either the "antigen" A or the foreign protein used as "Schlepper."

Since the number of suitable livers from patients dying of cancer and the limited yield of active material obtained from these livers was insufficient for our studies, other possible sources were investigated.

The analogous fractions from bovine liver, hypophysis, suprarenals, and gonads gave unsatisfactory results. Previous experience with deoxycholic acid and methylcholanthrene as possible antigens indicated

[1] Received for publication June 25, 1954.

[2] Presented at a meeting of the American Association for Cancer Research, Atlantic City, N. J., April 10, 1954.

[3] This work was supported in part by the Jewish Fund for Medical Research—Trattner Foundation, and by a grant-in-aid from the U. S. Public Health Service.

[4] The authors wish to express their appreciation to Dr. A. J. Haagen-Smit, Professor of Biochemistry, and to Dr. Dan Campbell, Professor of Immunochemistry—both of the California Institute of Technology, Pasadena, Calif.—for their valuable assistance and co-operation.

[5] This paper deals with four different "antigens," designated as follows: A denotes a nonsaponifiable liver fraction; B a bile-acid (oil) derivative, about 80 percent of which on analysis consisted of ethyl ester of choladienic acid; C a crystalline material, presumably ethyl choladienate, produced by Dr. Haagen-Smit, M. P. 38° C.; and D a crystalline material designated as ethyl choladienate, produced by the American Cyanamid Company, M. P. between 70° and 71° C.

some serologic differences between sera from normal individuals and from patients with cancer. Consequently the possibility that sterols, such as cholesterol, cholic and deoxycholic acids, or their derivatives, might react similarly to the liver fraction suggested itself. These sterols proved to be serologically inactive. In a study of their dehydration products, however, it was found that either the ethyl or methyl ester of choladienic acid, or the methyl (and probably the ethyl) ester of cholatrienic acid, could function as a serologically active antigen.

Preliminary studies were made in an effort to determine the serum components or component that might be involved in the flocculation reaction. Some of these studies were made in Professor Campbell's laboratory (*14*) and some in our own with "antigen" D. Fractionation of the sera was carried out by precipitation with increasing concentrations of ammonium sulfate at *p*H 7.8. The resulting fractions were then characterized by electrophoretic analysis and their serologic activity determined by flocculation tests with our standard methods. Fractions were obtained at ⅓, ½, and ⅔ saturation with salt. The first contained all the *gamma* globulin and some *beta*, the second contained the rest of the *beta* and all the *alpha*-2, the third contained all the *alpha*-1 globulin and traces of albumin, while the final soluble fraction contained the remaining albumin. The concentration of protein used for the flocculation tests was always the same as in the original serum, since the volume of the protein solution was adjusted to that of the original serum by the addition of physiological saline. The results consistently showed that the flocculation activity was associated with the fast-moving components and principally with the *alpha*-1 fraction. Although the albumin components showed no activity by themselves, they increased the sensitivity of the *alpha*-1 when recombined with it. This finding furnished the basis for the addition of bovine-serum albumin to the standard flocculation system. Similar fractionation and analysis of false positive sera gave essentially the same results. Although the immunological significance of the serologically active component is questionable, it is of interest that Campbell *et al.* (*15*, *16*) found that the reaginic antibodies in allergies often occurred in the *alpha* fractions. Roberts (*17*) also reported a significant increase of both *beta* and *alpha* fractions in lymphosarcoma.

Materials and Methods for Preparation of "Antigen" B

Ten grams of deoxycholic acid (Fisher Scientific Company)—purified in our laboratory according to the method of Reichstein and Sorkin (*18*)—were dissolved in 50 ml. of absolute ethanol in a 500-ml., round-bottom three-necked flask. The flask was then placed in an ice bath.

One hundred ml. of concentrated sulfuric acid, refrigerated at −5° to −10° C. was placed in a separatory funnel and introduced dropwise, under nitrogen, with constant stirring, into the deoxycholic acid-ethanol solution. The temperature, controlled by the rate of flow of the H_2SO_4, was allowed to rise rapidly to 38° C. and was maintained between 38° to 42° C. It is important to note that temperature below 37° C., or above

50° C., will result in an unsatisfactory yield. After the addition of the sulfuric acid had been completed, the solution was placed in a refrigerator at approximately 4° C. At the end of 18 to 20 hours, the reaction mixture was added slowly to about 1 liter of cracked ice and transferred to a 2-liter separatory funnel. When cool, the suspension formed was extracted once with 600 to 800 ml. of *n*-hexane (Phillips). The aqueous phase was discarded. The *n*-hexane solution was washed with several 250-ml. portions of distilled water until it cleared. The *p*H of the final washings must be about 6. The *n*-hexane solution was then mixed with 3 to 4 200-ml. portions of 5-percent solution of anhydrous Na_2CO_3.[6] A clear aqueous phase of the Na_2CO_3 solution indicated that the extraction was completed. The *n*-hexane was washed with distilled water until neutral to nitrazine paper and dried over Na_2SO_4. After filtering, most of the solvent was removed by distillation over a steam bath. The residual yellow viscous oil (7.0 to 8.5 gm.) was then dried *in vacuo*.

The desired fraction was isolated by chromatographic separation on activated alumina (Harshaw Scientific, Division Harshaw Chemical Co.).

The chromatographic equipment employed was similar to the one recommended by Zeichmeister and Cholnoky (*19*). It consisted of a column 30 mm. (inside diameter) by 800 mm. with standard taper joints at each end, separatory funnel to fit the top, and a perforated glass top. The chromatogram was prepared as follows:

The alumina was weighed in a dry beaker in the ratio of 30 grams of the adsorbent per gram of material to be chromatographed. The base of the column was covered with a thin pledget of absorbent cotton. With the finger securing the outlet, the chromatographic tube was half filled with *n*-hexane. The alumina was then introduced by continuous pouring and the opening of the outlet was simultaneously released. The lower end of the tube was tapped gently with a rubber hammer until the alumina was evenly settled. A thin pledget of absorbent cotton was then pressed gently over the top of the alumina.

From this point on the rest of the procedure was carried out in a dark room. The residue obtained from the sulfuric-acid treatment was dissolved in 150 ml. of *n*-hexane, poured into the column, and allowed to descend by its own gravity. The chromatogram was further developed by additional *n*-hexane and treated as a "fluid column." At the end of 200 ml. of hexane, the column under fluorescent light (Mineralight Model, S.L., 3,500 to 4,000 Å, Ultra-Violet Products, Inc., South Pasadena, Calif.), reading from above downward, presented the following zones:

(*A*) An olive-green to yellow zone (band) measuring	30	mm.
(*B*) A yellow-range zone	7	"
(*C*) A blue fluorescent zone	55	"
(*D*) A purple zone	15	"
(*E*) Two narrow blue fluorescent zones, almost confluent	2	"

[6] An active fraction could be obtained from Na_2CO_3 solution by acidification, extraction with n-hexane, followed by chromatography as described. The yield, however, was relatively small.

344699—55——25

The column was further developed with additional *n*-hexane and frequently observed with ultraviolet light until zone *E* entered the filtrate. Receiving flasks were then changed and the succeeding eluate, which contained the active fraction, was collected. The elution was continued until most of zone *D* was in the filtrate (about 2.0 to 2.5 liters). The elution end point was determined by the appearance of an olive-green color in the Tortelli-Jaffé reaction (*20*) performed on a small portion of the filtrate. The eluate containing zone *D* was distilled off a steam bath at reduced pressure. The oily residue (2.5 to 3.0 gm.) was dried *in vacuo* and redissolved in about 75 to 100 ml. of fresh *n*-hexane.

The material remaining on the column was found to be serologically inactive.

For purification, the eluted zone *D* was rechromatographed on activated alumina in the ratio of 20 grams of adsorbent per gram of the oil in the solution. A chromatographic tube measuring 20 mm. (inside diameter) by 425 mm. was employed. The procedure previously outlined was repeated. If zone *E* reappeared at the bottom of the column, it was eluted and flasks changed. Elution was continued with another 300 to 400 ml. of *n*-hexane to obtain active material from zone *D*. The solvent containing this fraction was removed at reduced pressure over a steam bath. The residue ("antigen" B), a clear, colorless, viscous oil (1 to 1.5 gm.) was dried *in vacuo* and dissolved in 95-percent ethanol in a concentration of 20 mg. per ml. This solution was transferred to brown bottles with airtight caps and stored under nitrogen at about 4° C.

Among the difficulties initially encountered were lack of reproducibility and instability of the active fraction. These were overcome by the use of purified solvents as suggested by Doctor Haagen-Smit. It should be noted that in the preparation of "antigen" B, either absolute methanol or absolute ethanol may be employed. In our experience, however, absolute ethanol produced a more satisfactory antigen. The activity of some of the later preparations has lasted for approximately 1 year.

The Chemistry of "Antigen" B

The compound is unstable in the dry state in the presence of air at 0° or 4° C. or at room temperature; it changes color and becomes serologically inactive. It has been found to be quite stable in ethanol solution at 0° or 4° C., provided the air in the container is replaced by nitrogen gas.

The preliminary investigations on the chemical nature of "antigen" B disclosed:

1) The material is a colorless, transparent, viscous oil which does not crystallize from alcohol, acetone, or ether.
2) It is very soluble in most organic solvents, readily soluble in ethanol, less soluble in methanol, and insoluble in water.
3) The oil ("antigen" B), obtained by the treatment of the deoxycholic acid with H_2SO_4, results presumably in the removal of 2 OH groups, as indicated by the determinations of C and H calcu-

lated for $C_{26}H_{40}O_2$. Analysis of 3 samples of questionable purity revealed the compound to contain the following:

Sample		*Carbon*	*Hydrogen*
1	:	80.02%	10.38%
2	:	82.11%	11.42%
3	:	79.91%	11.05%

4) It absorbed nearly two moles of hydrogen (1.82) on catalytic hydrogenation with platinum oxide in glacial acetic acid, suggesting the presence of two double bonds.
5) The compound did not react with semicarbazide or 2,4-dinitrophenylhydrazine. It did not form a picrate.
6) Refraction and density determinations showed:

$d^{25.5}$	1.0257
N_D	1.5340

7) The infrared absorption spectrum showed significant peaks at 5.75 mμ (1,739 cm.$^{-1}$), 9.07 mμ (1,120 cm.$^{-1}$), 9.66 mμ (1,035 cm.$^{-1}$). The 5.75 mμ peak indicates an ester or conjugated carbonyl function and makes the presence of a lactone structure unlikely. The 9.07 mμ peak has been attributed by Josien *et al.* (*21*) to —C—O—CH_3 stretching, and the 0.66 mμ peak is a typical —C—O frequency. These peaks are consistent with an ester grouping.

The results of these analyses suggested that "antigen" B consisted chiefly of the ethyl ester of choladienic acid. Investigation of the serologic behavior of this compound in its crystalline state seemed in order.

Preparation of "Antigen" C (Crystalline)

Doctor Haagen-Smit prepared the crystalline ethyl ester of choladienic acid according to the following procedure:

Thirty grams of purified deoxycholic acid (*18*) were heated for 1 or more hours *in vacuo* (0.004–0.01 mm.) with a Woods metal bath at 250 to 285° C. Nitrogen gas was bubbled over the deoxycholic acid through a capillary tube. After bubbling stopped (1–2 hours) or when no more water was evolved, the residual oil (orange-yellow colored) was distilled with 0.001-mm. pressure.[7] The flow of the distillate into the receiving flask may be facilitated by heating the neck of the distilling flask. A crystal-clear, plasticlike distillate was obtained in the receiving flask.

The distillate was dissolved in peroxide-free ethyl ether with slight warming and constant stirring. The ether was then extracted with 1 *N* KOH several times, washed to neutral with distilled water, and set aside. The ether contained the neutral pyrolyzed fraction which was serologically inactive.

The aqueous KOH phase was extracted three times with ether and then acidified to *p*H 2.0 with 4 *N* HCl. The resulting precipitate was extracted with ether. The ether was washed to neutral with distilled water and dried over anhydrous sodium sulfate for 30 minutes or more.

[7] A mercury pump was employed for these low pressures.

A sufficient amount of absolute methyl alcohol was added to dissolve the oil. The solution was then placed in an ice-salt bath until the choladienic acid crystallized. The white crystals were filtered, washed once with cold methyl alcohol, and dried *in vacuo*.

The choladienic acid (15–18 gm.) was recrystallized from absolute methyl alcohol. (Melting point of the crystals of choladienic acid was found to be 130–133° C.)

The choladienic acid was dissolved in absolute alcohol with a few ml. of concentrated sulfuric or hydrochloric acid (1 part choladienic acid to 20 parts of alcohol). This solution was kept at room temperature overnight (18–24 hours), after which distilled water was added to dilute the alcoholic solution to, approximately, a 70-percent concentration. Barium carbonate in sufficient quantity was added to remove the sulfate ions. The alcohol was removed by distillation under reduced pressure. The residual turbid aqueous phase was extracted three times with peroxide-free ethyl ether. This ether phase was washed with distilled water till neutral, then extracted several times with 1 *N* sodium carbonate. The ether phase was again washed till neutral and dried over anhydrous sodium sulfate.

The ether was removed by distillation and the oil (crude ethyl ester) dried *in vacuo*. The oil was distilled *in vacuo* (0.001–0.01 mm.) with a Woods metal bath at 235 to 255° C. The clear distillate was dissolved in a sufficient amount of absolute methyl alcohol and cooled (in ice bath with brine). The white crystals formed were filtered in the *cold* room (0–4° C.). The crystals were redissolved in hot, absolute methyl alcohol and allowed to recrystallize overnight at 0 to 4° C. The flaky white crystals were filtered in the *cold* room (0–4° C.), collected and dried *in vacuo*. Approximately 50 to 65 percent (yield 7–10 gm.) of the ester has been obtained from the choladienic acid. The melting point of the ethyl choladienate thus prepared was 38.0° C. This material has since been refined and employed in our evaluation as "antigen" D.

"Antigen" D, presumably "ethyl choladienate," used in the subsequent investigations was supplied by the Research Division, Lederle Laboratories, Division American Cyanamid Company, Pearl River, N. Y., and is most probably a mixture of ethyl $\Delta^{2,11}$- and $\Delta^{3,11}$-choladienate. The material had a melting point of 70 to 71° C.,[8] and an optical rotation of $[\alpha]_D^{25°} + 30$ (chloroform). Its elemental analysis was as follows: Calculated for $C_{26}H_{40}O_2$: C, 81.20; H, 10.48. Found: C, 81.50; H, 10.81. Infrared absorption and analysis showed the presence of double bond and ester groupings, and the absence of hydroxyl grouping.

Standardization of "Antigen" B for the Seroflocculation Reaction

The following procedure was employed for the standardization of "antigen" B.

Several sera were obtained from a number of cancer-bearing patients, designated as "positive" sera. Each of these was individually tested

[8] Comparative values for the melting point of this compound was unavailable as far as the literature could be traced.

by an antigen arbitrarily accepted as a "standard." (A standard "antigen" is one that had been previously tested on 48 sera and found to give over 95-percent negative results on the sera from 20 "normal" individuals, and over 95-percent positive results on the sera from patients known to have cancer. The sera from 8 patients having diseases other than cancer, such as rheumatoid arthritis, tuberculosis, etc., were included to complete the 48 sera. The only purpose of including sera from patients wih diseases other than cancer was to give us some idea as to how the "antigen" might be expected to behave under such circumstances. The positive sera were pooled and diluted 1:8 with 0.6-percent saline (*p*H 6.0–6.1) buffered with acetic acid and sodium acetate (*22*). One-half ml. aliquots of the diluted serum were placed in test tubes (13 × 100 mm.), stoppered with corks and quick-frozen in a dry ice-acetone mixture. These tubes were then removed immediately to a deep freeze at −10° C. The negative sera were treated in an identical manner. To test the activity of a newly prepared bile-acid derivative, a positive and a negative diluted frozen serum were employed. Each tube was used only once.

The Serologic Procedures

Preparation of sera.—Samples of blood were collected in the usual manner, obtained by venipuncture under aseptic conditions and employing a dry syringe. After clotting, the serum was separated and inactivated in the water bath at 56° C. for 30 minutes to remove interfering action of complement. Should it be desired to test any particular serum on subsequent days, we have found it necessary to re-inactivate it for 10 minutes at 56° C. for the second and third runs. Subsequent tests required no additional inactivation.

Preparation of solutions:

I. *Buffer solution*

The buffer solution is prepared by mixing 12.5 ml. of 0.2 *M* H_3CCOO Na and 12.5 ml. of 0.3 *M* H_3CCOOH, and diluting to 250 ml. in a volumetric flask (*22*).

II. *Saline solution*

To prepare the acetate buffered saline solution, add 10 ml. of the buffer solution I to 100 ml. of 0.6% saline. This mixture should give a *p*H of 6.0 to 6.1.

III. *Tween 80 and bovine albumin stock and working solution*[9]

a) Make a 1% Tween 80 solution using acetate buffered saline (II).

b) Make a 5% bovine albumin solution using the same diluent as (*a*).

c) Mix 0.1 ml. of (*a*) with 10 ml. of (*b*).

d) Mix 0.3 ml. of (*c*) with 100 ml. of the acetate buffered saline (II), adjust *p*H to 5.6 ± 0.2. This solution should be freshly prepared every week and the *p*H checked daily before using.

Procedure for the Seroflocculation Reaction Using "Antigen" B

The reagents, solutions, and equipment used in the seroflocculation reaction employing the bile-acid derivatives are similar to those previ-

[9] The functions of Tween 80 (Atlas Powder Company, Industrial Chemicals Department, Wilmington, Del.) and bovine albumin (Armour Product) in this procedure are to enhance the stability of the antigen suspension.

ously reported (*1*). The modifications in the antigen-serum ratio were the results arrived at on a purely empirical basis after numerous experiments.

Procedure:

1) Set up 4 tubes (13 × 100 mm.) numbered 1, 2, 3, and 4 for each serum.
2) Employing a 1.0-ml. pipette, deliver 0.35 ml. of buffered saline into the row of tubes numbered 1, and 0.1 ml. into the row of tubes numbered 4.
3) Employing a Kahn pipette, deliver 0.025 ml. of the buffered saline to the bottom of the row of tubes numbered 2, and 0.05 into the row of tubes numbered 3.
4) Employing a 0.2-ml. pipette, for each serum deliver 0.05 ml. serum to the row of tubes numbered 1.
5) Mix serum and saline in tube #1 then deliver 0.1 ml. each into tubes numbered 2, 3, and 4, leaving 0.1 ml. in tube #1.
6) Mix tubes #2 and discard 0.025 ml.
7) Mix tubes #3 and discard 0.05 ml.
8) Mix tubes #4 and discard 0.1 ml.
   This leaves 0.1 ml. of solution in each tube; thus tube #1 contains a 1:8 dilution; tube #2, a 1:10 dilution; tube #3, a 1:12 dilution; and tube #4, a 1:16 dilution.
9) Prepare antigen suspension just prior to its use as follows:
   Into a clean test tube add in the following order:
   a) To the bottom of the tube add 0.018 ml. of 1% cholesterol in absolute ethanol.
   b) Also add 0.1 ml. of the 2% antigen solution in 95% ethanol (20 mg./ml. of alcohol). Mix by shaking in a 56° C. water bath for a few seconds.
   c) Then add 0.4 ml. of the acetate buffered saline solution containing Tween 80 and bovine albumin, *p*H 5.6±0.2 (solution III, *d*). Blow saline into the alcoholic solution and mix *thoroughly* to insure a homogenous emulsion.
10) Pipette immediately 0.1 ml. of antigen suspension into each of the tubes numbered 1, 2, 3, and 4.
11) Shake the rack by hand 2 to 3 times, then place in a Kahn shaker for 3 minutes (use interval timer).
12) Pipette 0.8 ml. of the buffered saline to each tube.
13) Centrifuge the tubes for 10 minutes at 3,000 r.p.m. (approximately 1,850 × g. International Centrifuge #2, conical head accommodating sixty 15-ml. centrifuge cups).
14) Read in a standard source of diffuse light after mild agitation or filliping. Observe for particles plus clearance of suspension.
15) Reading of the results:
   a) *Positive* (+)=Particles plus complete clearance in 1 or more tubes.
   b) *Negative* (−)=Complete turbidity with or without particles. A known positive and a known negative should be tested with all unknown sera.

Procedure for the Seroflocculation Reaction Employing "Antigen" D

All reagents and equipment employed are similar to the 4-tube method, except for the changes indicated below. The following procedure was found most satisfactory when employing the crystalline material ("antigens" C and D).

Procedure:

1) Set up desired number of test tubes (13 × 100 mm.) in a rack, 2 tubes per serum—properly labeled and numbered 1 and 2.
2) Pipette to the bottom, 0.03 ml. of serum to tube #1 and 0.02 ml. to tube #2.

3) To each tube add 0.075 ml. of antigen suspension. This is prepared as follows:
   a) Pipette 0.013 ml. of 1% cholesterol in absolute ethanol to the bottom of a clean test tube.
   b) Then add 0.1 ml. of 1.5% ethyl choladienate in 95% ethanol (15 mg./ml. of alcohol). Shake tube.
   c) To this ethanol mixture is added 0.4 ml. of solution III, *d* (page 231). *Mix thoroughly.* Pipette the antigen suspension immediately, and not later than 10 minutes after preparation.
4) Shake rack by hand 3 or 4 times; then shake in Kahn shaker for 3 minutes (use interval timer).
5) Dilute with 0.8 ml. of the acetate buffered saline (*p*H 6.0 ± 0.1).
6) Centrifuge the tubes for 10 minutes at 2,700 to 2,800 r.p.m. (approximately 1,800 × g. International Centrifuge #2, conical head accommodating sixty 15-ml. centrifuge cups).
7) The interpretation of the results is similar to the 4-tube procedure.

Reagents

1) Deoxycholic acid, C. P., Fisher Scientific Company; purified by the method of Reichstein and Sorkin (*18*).
2) Ethyl ether, A. C. S., Mallinckrodt, treated with and distilled over ferrous sulfate ($FeSO_4$), calcium hydroxide (CaOH), and distilled water to remove peroxides.
3) 1 *N* Potassium hydroxide, A. C. S., Merck.
4) 4 *N* Hydrochloric acid, A. C. S., Mallinckrodt.
5) Absolute ethyl alcohol, U. S. Industrial Chemicals Company.
6) Methyl alchohol, absolute, A. C. S., Merck.
7) 1 *N* Sodium carbonate, A. C. S., Industrial Distributors, Inc.

Discussion

While not conclusive, the data previously presented (*1*) suggest that the flocculation reaction obtained using the liver fraction ("antigen" A) may conceivably be an antigen-antibody complex. The flocculation phenomenon produced by "antigens" B, C, and D is possibly analogous in character, although further studies are necessary to determine whether the bile derivatives react by identical or similar mechanisms. Should this be the case, it is conceivable that chemically related degradation products, with possible carcinogenic and/or antigenic capacities, may be evolved from steroids as a result of metabolic disturbances. On the other hand, the occurrence of the flocculation reaction with the bile-acid derivatives may be a cross-reaction phenomenon.

In a previous communication (*23*), it was suggested ". . . that an excess of cholesterol or its decomposition products may interfere with normal cell function. As a result of this interference . . . the cell will either multiply or die." Attention was also called (*24*) to the hypothesis ". . . that many forms of cancer may originate in the metabolic production of methylcholanthrene or related substances from the bile acid."

Similar views were later expressed by other investigators; Fieser (*25*) stated, "The most potent carcinogenic hydrocarbon yet known was

first obtained as a degradation product of a bile acid. There is some possibility that hydrocarbons of this type may be formed in the body." And again (*26*), "We know that methylcholanthrene can be produced in the laboratory by the degradation of deoxycholic acid, of cholic acid, and of cholesterol. Conversion under physiologic conditions has never been demonstrated, but one can say that it is at least conceivable that carcinogens under certain conditions of abnormal metabolism may arise in the body and give rise to cancer." Greenstein (*27*) advanced the opinion, "Although a polycyclic hydrocarbon such as methylcholanthrene has been considered as an extrinsic agent, it is possible to conceive of this substance as arising by a process of abnormal metabolism from the bile acids, and, hence, under such conditions to be classed as an endogenous agent." Boyland (*28*) pointed out, "Another aspect of the metabolism in relation to cancer is the possible formation of polycyclic carcinogenic hydrocarbons from hydroaromatic substances such as sterols."

In the light of these hypothetical considerations, the possible carcinogenic properties of the bile-acid derivatives should be investigated. Study of the flocculation reaction with the aid of these derivatives may permit a deeper insight into the serologic mechanism of this phenomenon and perhaps its ultimate metabolic significance.

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The Role of Bile-Acid Derivatives in the Seroflocculation Reaction. II. Clinical Evaluation[1,2,3]

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This communication concerns the clinical evaluation of a seroflocculation reaction in health and disease after use of a bile-acid derivative as an "antigen." This derivative employed functions as a substitute for the lipoid fraction previously described and reported.

The authors reported in 1951 (*1*, *2*), that an "antigen" A, prepared from the livers of individuals who succumbed to cancer, yielded a positive reaction in 98 percent of instances when mixed with the sera from patients with untreated invasive cancer. From 1944 to 1954 a total of 10,830 reactions have been recorded, consisting of sera from 3,025 patients with histologically positive cancer, 4,244 individuals with nonmalignant disease, and 3,561 "normal" individuals (table 1).

The various "antigens" employed in this paper are designated and defined as follows:

"Antigen" A—the nonsaponifiable fraction obtained from the livers of patients dying of cancer.

"Antigen" B—oil preparation prepared from deoxycholic acid, the active principle considered to be ethyl choladienate.

"Antigen" C—first crystals prepared by Haagen-Smit and considered to be ethyl choladienate.

"Antigen" D—commercially supplied as ethyl choladienate.

The use of "antigen" A is of academic interest and has a limited applicability because of the following factors: 1) Fresh unembalmed human liver is difficult to procure in sufficient amount to obtain the desired amount of "antigen" material. 2) Preparation procedures are technically complicated and time-consuming. 3) The expense of production is pro-

[1] Received for publication June 25, 1954.
[2] Presented at a meeting of the American Association for Cancer Research, Atlantic City, N. J., April 10, 1954.
[3] This work was supported in part by a grant-in-aid from the U. S. Public Health Service.
[4] From the Veterans Administration Hospital Center (Tumor Control Center), and the Department of Radiology, University of California Medical Center, Los Angeles, California.

TABLE 1.—*Seroflocculation reaction (1944 to 1954)*

Date	Laboratory	Total reactions	Cancer sera (*number*)	Positive (*percent*)	Nonmalignant sera (*number*)	Positive (*percent*)	Normal sera (*number*)	Positive (*percent*)	"Antigen"
1944–1945	Dr. Penn—Private	1, 701	238	81. 0	471	20. 0	992	1. 1	A
1945	L. A. County Hosp.—Dr. Butt	250	111	81. 1	139	31. 6	0	0	A
1948	Calif. Inst. of Tech.—Dr. Campbell	157	55	89. 1	102	31. 37	0	0	A
1948	Birmingham Gen. Hosp. (Los Angeles), V. A.—Dr. P. W. West	157	55	85. 46	102	33. 33	0	0	A
1948	V. A. Center—Authors	157	55	90. 9	102	22. 54	0	0	A
1948–1950	V. A. Center—Authors	4, 263	830	98. 6	2, 435	12. 7	998	0. 9	A
1951	V. A. Center—Dr. Byron	500	185	98. 38	315	28. 57	0	0	A and B
1951	V. A. Center—Dr. Mumma	552	465	98. 0	87	9. 2	0	0	A and B
1953	L. A. County Hosp.—Dr. Butt	120	33	93. 4	37	9. 6	50	4. 0	B
1950–1954	V. A. Center—Authors	2, 973	998	94. 78	454	14. 59	1, 521	2. 8	B and C
Total		10, 830	3, 025		4, 244		3, 561		

hibitive. 4) Yields of active material obtainable are low. 5) Stability of "antigen" is unpredictable and indefinite.

It was then necessary to investigate various other biological sources in search of a suitable substance to be used as an "antigen." As described in paper I (p. 225), it was found that the substance, presumably ethyl choladienate, prepared from deoxycholic acid functioned satisfactorily as a serologically active fraction when used as "antigens,"—designated "antigens" B, C, and D in this communication.

The clinical evaluation of this phenomenon has been carried out by our group in the Tumor Control Center of the Veterans Administration Hospital Center, Los Angeles, California, under grants-in-aid to the Department of Radiology, School of Medicine of the University of California at Los Angeles. Histopathologic diagnoses were obtained as a routine procedure from the Pathology Department, under the direction of Leo Kaplan, M.D., Veterans Administration Hospital Center, Los Angeles, California. All determinations reported in this paper were made on unknown sera. The sera were designated by number as the only means of identification. The clinical records of all diseased patients were screened by a physician. The presence or absence of cancer was determined by microscopic examination of tissues removed either at biopsy, operation, or autopsy. A further careful review was made of the clinical and laboratory records in regard to temperature, transfusions (1 week prior to testing) (*3*), drug ingestion (*4–7*), trauma (*8, 9*), and other forms of treatment such as irradiation (*10–17*). The only cases herein reported are those which were tested prior to any form of treatment. Complete record forms of each malignant and each nonmalignant disease case were filled out by a screening physician (table 2). Forms for "normal" individuals were filled out by the person at the time the blood was drawn (table 3). Individuals whose questionnaires were checked "Yes" in answer to the presence of existing disease or trauma were subsequently screened by a physician. Any individual on medication or treatment was placed in a separate category and therefore not considered "normal." Both males and females of various age groups were studied. The clinical records of the patients with nonmalignant disease were reviewed by a physician for the established diagnosis, clinical and laboratory status, and complicating factors which may have influenced the test. The cases were then placed in their respective classifications, regardless of the results of the test.

Using "antigens" B and C derived from bile acids, whose active principle is considered to be ethyl choladienate, 2,973 determinations were made (table 4). In 1950, our group was able to produce from deoxycholic acid an active oil "fraction" that remained stable from 48 to 72 hours. By refinement in the chemical procedures an active oil "fraction," "antigen" B, having a stability of 175 to 200 days, was produced. Further refinement in procedures by Doctor A. J. Haagen-Smit of the California Institute of Technology resulted in an active crystalline preparation, "antigen" C, which has remained stable for over 1 year (M. P. 38° C.).

TABLE 2.—*Nonmalignant and malignant questionnaire*

Hospital: _____ Location: _____

SERUM FLOCCULATION TEST
NONMALIGNANT AND MALIGNANT CASES

Date blood was taken	Patient File Number	Serum Number
Name - last first middle	Address	
Age / Sex / Race	Height _____ feet _____ inches	Weight

A. Has the patient had any of the following diseases in the last year?

	Yes	If yes, date		Yes	If yes, date		Yes	If yes, date
Hay Fever			Asthma			Active Tuberculosis		
Rheumatism			Arthritis			Syphilis		

Give details for each disease checked:

AT THE TIME BLOOD WAS TAKEN

B. What was the patient's highest temperature on date of test?

C. What medication was the patient getting at that time? (State drug, dosage and duration)

D. Was there any recent (10 days) trauma (accident or surgery)? If yes, give date and describe:
☐ Yes ☐ No

E. Had the patient had recent (10 days) transfusions? If yes, give date and amount:
☐ Yes ☐ No

F. IF THE PATIENT IS FEMALE

Was there any vaginal bleeding at the time blood was taken? ☐ Yes ☐ No

If yes, was there excessive bleeding? ☐ Yes ☐ No

Was she pregnant? ☐ Yes ☐ No

If yes, date of last menses

FINAL DIAGNOSES (For non-cancer patients)

CANCER PATIENTS ONLY

A. AT THE TIME BLOOD WAS TAKEN, what definitive treatment was being given this patient's tumor?

☐ New tumor, never treated
☐ New tumor, under treatment
☐ Recurrent tumor, not being treated
☐ Recurrent tumor, under treatment

TYPE OF TREATMENT
☐ Surgery
☐ Radiation
☐ Other, specify: _____

INTENT OF TREATMENT
☐ Curative
☐ Palliative

B. Size of tumor, greatest diameter (cm.) if measurable
_____ cm.

C. Extent of disease
☐ Localized to tissue of origin
☐ Local spread (surrounding tissue and/or regional lymph nodes)
☐ Distant metastases to: _____

D. Basis of Diagnosis

Not based on tissue findings, was based on:
☐ Clinical findings ☐ Cytology
☐ X-ray ☐ Other

Histopathological
_____ Date
☐ Biopsy ☐ Autopsy
☐ Surgical specimen ☐ Other

E. Final Diagnosis

Primary site / Cell type / Grade of malignancy

Results	Titer	Fractionation
☐ Positive ☐ Negative		☐ Positive ☐ Negative

State of California
Department of Public Health

10-1-54
Form PD-1033

TABLE 3.—*"Normal" individual questionnaire*

Hospital: _____ Location: Los Angeles

SERUM FLOCCULATION TEST
"NORMAL" INDIVIDUALS

Date blood was taken	Record Identification	Serum Number
Name - last first middle .	Address	
Age / Sex / Race / Height _____ feet _____ inches / Weight		
Occupation		

	Yes	No	If yes, when? Month Year
1. Do you have now or have you had in the last year:			
a. An attack of hay fever			
b. An attack of asthma			
c. Rheumatism or arthritis that incapacitated you			
d. Active tuberculosis of the lung			
e. A diagnosis of syphilis			
f. An incapacitating injury or accident			
g. A transfusion			
2. Have you had surgery within the last 3 months?			
If yes, what was done?	xx	xx	xxxxxxxxxx
3. Are you now under a doctor's care for treatment?			
If yes, state illness:	xx	xx	xxxxxxxxxx
4. Are you taking any medicine, by mouth or by injection?			
If yes, name the medication:	xx	xx	xxxxxxxxxx
5. FOR FEMALES			
a. Are you menstruating now?			xxxxxxxxxx
b. Are you pregnant?			xxxxxxxxxx
If yes, date of last menstrual period	xx	xx	

Results	Titer	Fractionation
☐ Positive ☐ Negative		☐ Positive ☐ Negative

State of California
Department of Public Health

10-1-54
Form CD-1834

Our most recent crystalline preparation, "antigen" D, considered to be ethyl choladienate, is prepared by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. (Details of "antigen" D, see paper I, p. 225.)

TABLE 4.—*Determinations—"antigens" B and C (1950 to 1954)*

"Normal" healthy individuals	1, 521
Histologically positive cancer cases	998
Nonmalignant, hospitalized pathologic cases	454
Total determinations	2, 973

A comparative study was made with "antigens" B and C on the same sera. Of the 170 cases of cancer thus studied there was no significant difference in the percent-positive reactions of the two fractions (table 5). A similar comparison was found in a series of 268 "normal" sera (table 6). In 73 hospitalized individuals with nonmalignant disease, the crystalline "antigen" C revealed fewer false positive reactions than did "antigen" B (table 7).

TABLE 5.—*Carcinoma determinations—comparison of "antigens" A and B*

Site of malignancy	Total reactions	"Antigen" B		"Antigen" C*	
		Positive reactions	Negative reactions	Positive reactions	Negative reactions
Head and neck	41	38	3	39	2
Chest	37	36	1	36	1
Gastrointestinal	31	30	1	30	1
Genitourinary	26	24	2	25	1
Bone	10	9	1	9	1
Melanoma	3	2	1	2	1
Basal-cell epithelioma	12	11	1	11	1
Squamous-cell epithelioma	1	1	0	1	0
Lymphoma	6	4	2	4	2
Breast	3	3	0	3	0
Total reactions	170	158 (92. 95%)	12	160 (93. 75%)	10

*Melting point 38.0° C.

TABLE 6.—*Determinations from "normal" healthy individuals—comparison of "antigens" A and B*

Group	Total reactions	"Antigen" B		"Antigen" C*	
		Positive reactions	Percent positive	Positive reactions	Percent positive
Age: 20–40 years					
Male	54	2	3. 7	1	1. 8
Female	65	0	0	1	1. 5
Age: 40–70 years					
Male	71	4	5. 6	2	2. 8
Female	78	2	2. 5	3	3. 8
Total reactions	268	8	2. 91	7	2. 61

*Melting point 38.0° C.

TABLE 7.—*Nonmalignant determinations—comparison of "antigens" A and B**

Diagnoses	Total Reactions	"Antigen" B		"Antigen" C†	
		Positive	Negative	Positive	Negative
Rheumatoid arthritis	8	4	4	4	4
Active TBC	8	3	5	2	6
Coccidiomycosis	3	1	2	1	2
Lung abscess	2	0	2	0	2
Bronchial asthma (no medication)	15	1	14	0	15
Bronchiectasis	6	0	6	0	6
Gastric ulcer	4	1	3	1	3
Breast—benign	7	0	7	0	7
Bone cyst	6	0	6	0	6
Benign prostatic hypertrophy	7	2	5	0	7
Keratosis	7	0	7	0	7
Total reactions	73	12 (16.4%)	61	8 (10.95%)	65

*Hospitalized cases without cancer.
†Melting point 38.0° C.

The clinical evaluation of the present active crystalline material, "antigen" D, has been given intensive study during the past few months. This "antigen" is repeatedly reproducible and results in a consistent serologic response. A total of 268 sera, consisting of 103 "normal" individuals, 80 histologically positive cancer cases, and 85 individuals with nonmalignant pathology were studied (table 8). The sera from this group were first studied by the routine flocculation procedures, second by serum serial dilutions, and third by protein fractionation studies of those sera which reacted positively by the routine procedure.

TABLE 8.—*Determinations—"antigen" D*

"Normal" healthy individuals	103
Histologically positive cancer cases	80
Nonmalignant, hospitalized pathologic cases	85
Total determinations	268

"Normal" Healthy Individuals

In the group of "normal" healthy individuals 103 sera were obtained from employees of the Veterans Administration Hospital Center. These employees are required to have a yearly physical examination and, therefore, were assumed to be "healthy." The age groups and results of the serofloccculation reaction are shown in table 9. A personal screening, in addition to the questionnaire, was done on the 4 individuals giving a positive reaction. No causative factors could be found since physical examination and special diagnostic procedures were not routinely carried out.

TABLE 9.—*"Normal" healthy individuals ("antigen" D)*

Group	Total number of cases	Positive reactions	Remarks
Age: 20–40 years			
Male	22	2	No cause found*
Female	37	0	
Age: 40–70 years			
Male	21	1	No cause found*
Female	23	1	No cause found*
Total	103	4 (3.8%)	

*Four cases from history and clinical screening revealed no causative factors.

Cases of Verified Cancer

Eighty sera from individuals who had various types of malignancy were studied, as shown in table 10. It should be noted that in a Veterans Administration Hospital Center an abundant supply of breast and female pelvis malignancies is not available. In this group of 80 untreated cancer patients, 100 percent gave positive reactions; however, from past experiences and in larger group studies, the positive percentages have varied from 92 to 95 percent.

The clinical evaluation of any test procedure as an adjunct in the detection of cancer depends upon the earliest stage at which cancer can be detected. An arbitrary classification into 4 stages of all cancer cases studied is shown in table 10. The positive reactions obtained in the various stages indicate that approximately the same percentage of positive reactions will be found in each of our 4 clinical classifications.

TABLE 10.—*Cases of verified cancer ("antigen" D*)*

Site of malignancy	Total number	Clinical classification†			
		Stage 1 (*number*)	Stage 2 (*number*)	Stage 3 (*number*)	Stage 4 (*number*)
Head and neck	15	3	4	8	0
Chest	16	4	5	7	0
Breast	2	0	0	2	0
Gastrointestinal	9	0	2	5	2
Genitourinary	18	5	7	6	0
Bone	7	2	3	2	0
Lymphoma	1	0	0	1	0
Basal-cell epithelioma	6	6	0	0	0
Squamous-cell epithelioma	6	3	1	2	0
Total	80	23	22	33	2

**NOTE:* All sera showed positive reaction.

†Clinical classification of all cases of cancer having positive biopsies.

Stage #1 = Local lesions; no symptoms.

Stage #2 = Local lesions; symptoms; no metastases.

Stage #3 = Local lesions; symptoms; amenable to treatment.

Stage #4 = Generalized carcinomatosis; terminal palliative care only.

Our investigations have shown that the seroflocculation reaction *will not be positive* in the vast majority of instances of carcinoma *in situ*—even though a few instances have been found to result in positive reactions in carcinoma *in situ* of the stomach and larynx. In general, such noninvasive lesions yield negative reactions. In our experience, early invasive carcinomas (1 cm. in size), demonstrated by histologic examinations to have broken through the basement membrane, yield positive reactions in a high percentage of instances.

Nonmalignant Other Pathologic Conditions

The results on sera from individuals having disease other than cancer are tabulated in table 11. All cases except those of the menorrhagic group were hospitalized at the time the blood specimen was taken. This particular group of diseases was purposely selected because of its known proclivity to give a high percentage of positive reactions. Consequently the percentage of positive reactions in this particular group is high. It has been shown, during the past 6 years, that the over-all percentage of false positive reactions in noncancerous diseases will be from 12 to 20 percent when employing our various "antigens" or their modifications.

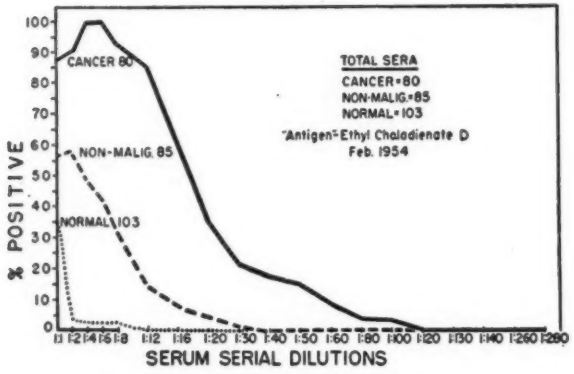
TABLE 11.—*Nonmalignant other pathologic conditions ("antigen" D)*

Pathologic conditions	Total number of cases	Positive reaction
Diabetes on insulin medication	14	11
Glandular dyscrasias on thyroid medication	10	8
Active rheumatoid arthritis	12	7
Active tuberculosis	12	5
Asthma after adrenalin medication	14	5
Menorrhagia after estrogenic medication*	10	6
Fever, 101°	2	2
Cirrhosis	2	2
Gastric ulcer	8	2
Multiple sclerosis	1	1
Total	85	49†

*Nonhospitalized. (All other cases hospitalized.)
†57.6% false positive reactions.

Serum Serial Dilution Study

The sera from these 268 patients were serially diluted independently. In our laboratory it has been shown that the technicians have difficulty in reading the flocculation reaction when the sera are diluted beyond 1:120. From text-figure 1 it is to be noted that all sera from the cancer patients resulted in a positive reaction in 85 percent of the cases in serum dilutions of 1:12. At this dilution, approximately 15 percent of sera from the nonmalignant pathologies are positive and 0 percent of positive reactions are found from "normal" individuals.



TEXT-FIGURE 1.—Serum serial dilutions.

Protein Fractionation of Serum

Preliminary investigations have been carried out to determine whether malignancies could be differentiated from non-neoplastic diseases by testing various fractions of the sera by the seroflocculation reaction. The ammonium sulfate method of Campbell and Bulman (*18*) was employed for this purpose.

Table 12 gives the results of this study. Fraction A consists of one-third saturated ammonium sulfate insoluble fraction; fraction B consists of one-half saturated ammonium sulfate insoluble fraction; fraction C consists of one-half saturated ammonium sulfate soluble fraction. Fraction B plus C in the malignant sera contained the active component in 97.0 percent of cancer sera. The active component in non-neoplastic diseases appeared dispersed in B plus C and A in approximately 1:1 ratio. The positive "normals" showed no reaction in either A or B plus C fractions.

TABLE 12.—*Results of the protein fractionation of positive sera*

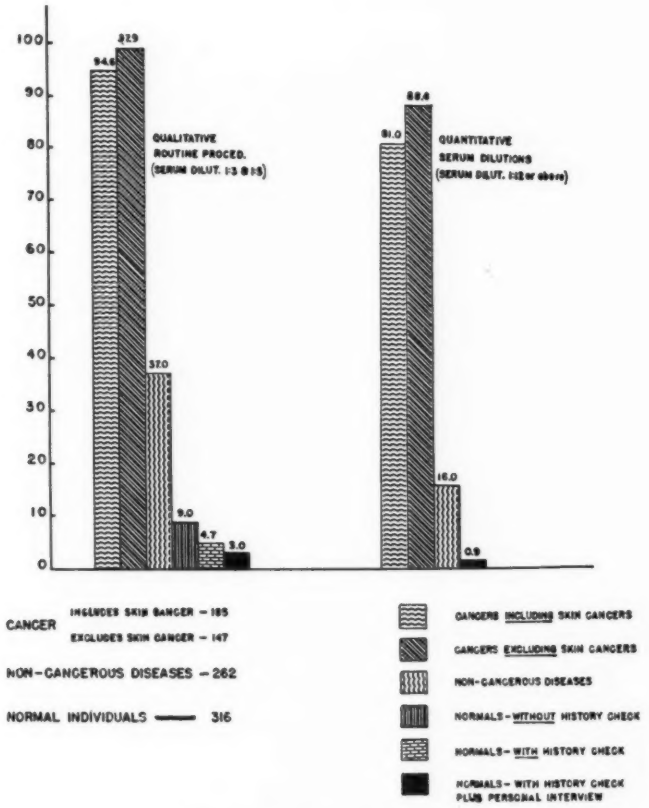
Total sera fractionated		Fractions*	
		A (*percent*)	B+C (*percent*)
Malignant neoplasms	33	27. 27	97. 00
Non-neoplastic diseases	25	52. 00	48. 00
Normals	4	0	0

*Interpretations: A, ⅓ saturated ammonium sulfate insoluble fraction; B, ½ saturated ammonium sulfate insoluble fraction; C, ½ saturated ammonium sulfate insoluble fraction. Results give percent-positive reactions in the seroflocculation test.

These preliminary data indicate, then, that it is possible to decrease the positive non-neoplastic diseases obtained in the qualitative reaction to 52 percent and the positive "normals" to a very low percent, since only sera positive by the routine method were fractionated.

A complete study of this phase of our work will be presented at a later date.

Additional studies of the seroflocculation reaction using "antigen" D are shown in text-figure 2. This group of sera was studied by 1) routine 2-tube qualitative procedure, and 2) quantitative serum dilutions. The 763 individuals consisted of 185 verified cancer cases of various stages and categories, 262 hospitalized noncancerous cases, and 316 "normal" persons.



TEXT-FIGURE 2.—Analysis of 763 seroflocculation results.

The seroflocculation reaction study, by routine 2-tube qualitative procedure, of 185 cancer sera, including skin cancers, gave 94-percent positive reactions. Excluding skin cancer, 97-percent positive reactions were obtained. By quantitative serum dilutions (1:12 or above) 81-percent positive reactions were obtained, including skin cancers, and 88-percent positive reaction results, with the exclusion of skin cancers.

Noncancerous diseases (262) gave 37-percent positive results by routine 2-tube qualitative procedures and 16-percent positive reactions by quantitative serum dilutions (below 1:12).

Of determinations of 316 "normal" sera, there were 28 positive reactions (9%) on routine 2-tube qualitative procedure—without checking questionnaire sheets filled out by each individual. All 316 questionnaire history forms were then checked by a physician. Only 13 individuals had checked an abnormal condition (disease or medication) on their questionnaire form, thus eliminating them from the "normal" group and reducing the percentage of false positive reactions to 4.7 percent. All positive reactors, however, were personally interviewed by a physician, and 5 additional individuals were found to have some abnormal condition or medication that they had failed to record on their history forms. Of the 10 remaining individuals (3.0%) showing positive reactions, no causative factors could be found. By quantitative serum dilutions below 1:12 the positive percentage was reduced to 0.9 percent.

Recently, we have studied 38 cancers of the skin in an attempt to correlate the size of the lesion with the seroflocculation reaction (table 13). One group consisted of 21 squamous-cell cancers of the skin, 10 of which were under 1.0 cm. in size. The serology was negative in 3 cases; each measured, respectively, 0.5, 0.5, and 0.8 cm. in diameter. Eleven lesions measured more than 1.0 cm. in size—of which 1 measured 1.5 cm. in size and showed a negative seroflocculation reaction.

TABLE 13.—*Cancers of skin*

Diagnoses	Total	Positive reaction	Percent positive reaction	Size of lesion		Negative reaction
				Under 1 cm.	Over 1 cm.	
Basal-cell epithelioma.....	17	14	82.3	7	10	3*
Squamous-cell epithelioma.	21	17	80.9	10	11	4†

*All over 1 cm.
†Three under 1 cm. (0.8, 0.5, 0.5); 1 over 1 cm. (1.5).

In addition, a second group of 17 basal-cell carcinomas of skin was similarly studied. Seven lesions were under 1.0 cm. in size, of which none was negative serologically. Ten cases had lesions over 1.0 cm. in size and of these 3 were serologically negative.

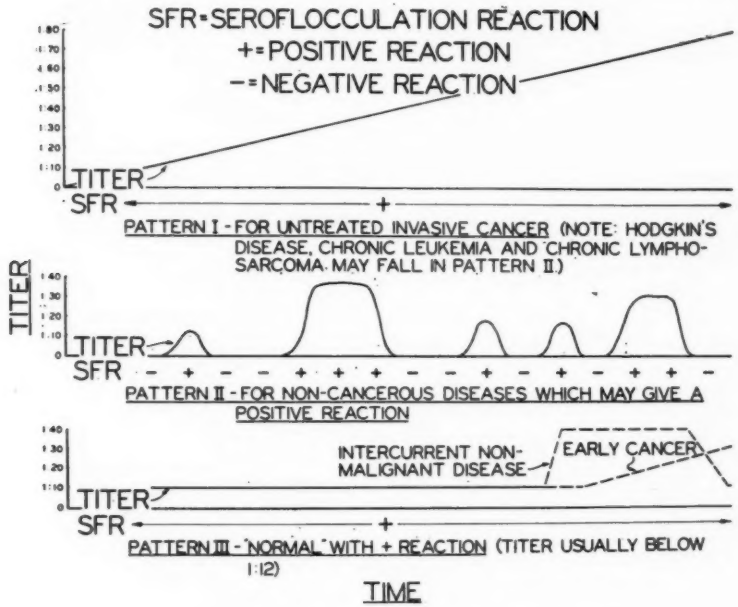
Hypothetical Curves of Titer Patterns

During the course of our studies it became apparent that three general types of curves would result (text-fig. 3), as noted in Patterns I, II, and III. These patterns were originally drawn as hypothetical curves. Our subsequent work tends to confirm this hypothesis.

Pattern I: In instances of untreated invasive cancer when it progresses to a given size (presumably at approximately 1 cm. in diameter) the

reaction becomes positive. The reaction should be repeatedly positive and the titer should rise with time. We have found that the titer will decrease and the reaction become negative in the terminal stage of the disease. A preliminary study of gastric cancer[4] tends to substantiate this pattern.

Pattern II: Most chronic diseases are self-limited or tend to undergo partial or complete remissions. In such instances the reaction has been found to be positive or negative, and the titer to fluctuate according to the degree of activity. Certain types of cancer, such as chronic leukemia, Hodgkin's disease, and lymphosarcoma, may fall into this pattern during



TEXT-FIGURE 3.—Hypothetical curves of titer patterns.

remission. It is equally possible that a few of the more progressive chronic diseases may likewise fall into Pattern I. In general, however, this should not be the case.

Pattern III: The few so-called "normal" individuals with a positive reaction have been found to have a low titer. This may be due to: 1) some temporary abnormality in steroid metabolism, 2) permanent disability in steroid metabolism, 3) some undetectable chronic disease, or 4) to a small undemonstrable cancer.

If the condition is a temporary abnormality, as in 1 above, the reaction should revert to negative on subsequent tests; if, as noted under 2 or 3

[4] The flocculation reaction is being studied in a series of gastric ulcers, some of which have, since the beginning of the study, been found to be gastric cancers.

above, the reaction may remain positive but at a low titer—unless the condition should progress to a more serious stage. If an acute episode such as acute infection supervenes, the titer would be expected to rise promptly and remain elevated during the acute phase, returning to its previous level on subsidence of the infection. On the other hand, if the positive reaction is due to a growing, invasive cancer at some point along the curve, we would anticipate a gradual rise in titer with time (table 13).

Discussion

Since 1948, materials employed by us in the seroflocculation reaction have gone through multiple modifications. We have designated these various substances as "antigens" A, B, C, and D.

"Antigen" A is the nonsaponifiable fraction obtained from the livers of patients who succumb to cancer. This "antigen" we believe to contain more than one compound, the active component or components of which have as yet not been identified and characterized. The reaction is nonspecific. We have no valid evidence that it is a true antigen-antibody response, though some of the characteristics of the "antigen" are compatible with this possibility. When one compares the serologic results of the various "antigens," one gains the impression that "antigen" A perhaps yields a lower percentage of positive results in the sera from noncancer patients and "normal" individuals.

"Antigens" B, C, and D are all derived from deoxycholic acid, and the active principle of each we believe to be ethyl choladienate. Support for this assumption must, at the present, rest upon the following documentation:

1) "Antigen" B was analyzed by Doctor Haagen-Smit and considered to consist mainly of ethyl choladienate.
2) On the basis of this, deoxycholic acid was pyrolyzed to produce choladienic acid. This in turn was ethylated to produce ethyl choladienate, or "antigen" C, which proved to give similar serologic results to "antigen" B.
3) We requested Lederle Laboratories Division, American Cyanamid Company to prepare for us crystalline ethyl choladienate. They have provided for us a limited amount of a crystalline substance ("antigen" D) which they have analyzed in their laboratory and consider to be ethyl choladienate (see paper I, p. 225).

"Antigens" B, C, and D give very similar serologic results in comparable percentages for untreated invasive cancer, certain nonmalignant diseases, and "normals"—with the exception that "antigen" D appears to give a higher percentage of positive results than do "antigens" B and C in the sera from patients with diseases other than cancer. This difference may be more apparent than real, however, since most of the patients reported in this group for "antigen" D were hospitalized for their disease, as contrasted with a similar group for "antigens" B and C where many of the individuals were ambulatory. The apparent difference in the percentage

of positive reactions noted could conceivably be a result of the extent or severity of the disease.

Under certain conditions, the test *per se* is not difficult to carry out; a good technician can master the procedures in a period of 2 weeks. There are, however, one or two critical points about the procedure which require caution. Without further comment, all analytical measurements must be done with great care when preparing the "antigen" emulsion. One of the most critical points is in maintaining a *stable* "antigen" emulsion with "antigen" D. This is dependent upon the proper addition of "antigen," cholesterol, Tween 80 (polyoxyethylene sorbitan mono-oleate), and bovine albumin, and maintaining the proper *p*H values, which may vary with temperature (see paper I, p. 225). With proper precautions the "antigen" emulsion will remain stable for 15 to 30 minutes. Any evidence of crystals settling on the sides of the tube containing the emulsion indicates that the "antigen" emulsion has broken down and should be discarded, and a new emulsion prepared.

Fresh serum must be inactivated at 56° C. for 30 minutes before initial testing. If serum is to be re-run after 8 hours of initial inactivation, it must be inactivated at 56° C. for 10 minutes. Two 10-minute inactivations at 56° C. have been found sufficient and no further inactivation is necessary.

In a Veterans Administration hospital the number of cancers of the breast, cervix, and uterus is extremely limited and therefore our results are, in general, based on cancers in the male. It is difficult to define early cancer since the term is subject to various interpretations and dependent, in part, upon numerous factors, such as carcinoma *in situ*, size and character of the primary tumor, presence or absence of metastasis, operability, and curability. In order to gain some rough idea of the serologic response to the extent of disease, we have arbitrarily classified the cancers in stages 1, 2, 3, and 4, as noted in table 10. We consider stages 1, 2, and 3 to be operable though not necessarily curable. In general, stages 1 and 2 should have the more favorable prognoses with the respective appropriate treatments. The size of the primary tumor would be expected to vary considerably, even in stage 1. Using this crude classification, however, we could find little, if any, difference among the 4 stages in the percentage of positive serology. Only occasionally did we find a positive reaction with carcinoma *in situ*. Exclusive of basal-cell carcinoma of the skin, it is our general impression that of the carcinomas we have studied the seroflocculation reaction will be positive in 90 percent of untreated invasive cancers which are 1.0 cm. or more in size.

Our studies to date would indicate that the seroflocculation reaction, when carried out in serum serial dilutions on all the sera positive by the routine method, may have clinical application in cancer and certain other diseases.

The serum from a patient with untreated invasive cancer should be repeatedly positive week after week. It is not unlikely the titer will rise with time in the earlier stages of cancer. We have noted these findings to be present in the sera of several patients on medical treatment for pre-

sumably benign gastric ulcers that later at operation proved to be gastric cancers. In one instance in which the cancer was thought to be completely removed at operation, a persistently preoperative positive reaction with a rising titer reverted to negative several days postoperatively. In two similar cases, the sera continued positive with a rising titer preoperatively. At operation extensive metastases were found. Sera determinations postoperatively continued to be positive but at a slightly lower level than prior to surgical intervention.

Most, but not all, of the nonmalignant diseases are self-limited in character and tend to have remissions. It would, therefore, be expected that the reaction of the sera from such individuals would fluctuate between remissions and recrudescence of the disease. Preliminary studies along these lines indicate this to be a reasonable assumption.

The sera from positive reactors among "normal" individuals may or may not be persistently positive, depending upon a demonstrable cause. If there is no obvious cause for the positive serology, the titer should remain at a low level, below 1:12, in all but exceptional instances.

Preliminary investigations with the serum-protein fractionation procedure suggest the reaction with "antigen" D to be mainly in the *alpha*-1 and *alpha*-2 fractions. While the present method of separation is qualitative rather than quantitative, it suggests that a more refined method may be helpful in eliminating a considerable percentage of the positive reactions in the sera from "normal" individuals and those having diseases other than cancer.

Our data indicate that the seroflocculation reaction, as herein employed, is measuring qualitatively and quantitatively a difference between the sera of most "normal" individuals and the sera of most individuals with cancer and other diseases. This difference is found most frequently, most persistently, and to a greater degree in the sera from cancer patients than in any other chronic disease which we have studied. The nature of this difference is as yet undetermined.

While the relationship of this reaction seems to be more clearly allied to the presence of cancer than to any other chronic disease, this correlation in itself is not of sufficient magnitude to make it a diagnostic procedure *per se*. Further studies should be directed toward the elucidation of the mechanisms involved and the determination of their role in those conditions which result in a positive serologic response.

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Proliferative Changes Induced in the Vaginae of Intact Strain BALB/c Mice with Croton Oil, Olive Oil, and Methylcholanthrene[1,2,3]

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Proliferative changes, normal to the vaginal epithelium of the mouse, have been described fully by Suntzeff *et al.* (*1*). These authors also observed that repeated and continued injection of an estrogen greatly increased this proliferation with the development, in some instances, of precancerous or carcinoma-like areas (*1*). The stimulating action of croton oil on the epithelium of the skin of the mouse has been described by numerous investigators (*2–4*). Since most studies employing croton oil have been confined to the skin, it appeared desirable to investigate what influence croton oil would exert on epithelial proliferation in the vagina. The influence of low doses of methylcholanthrene, or olive oil alone, or in combination with croton oil, also was investigated.

Materials and Methods

Cotton applicators or plugs immersed in olive oil alone, 1-percent croton oil[5] in olive oil, 0.1-percent methylcholanthrene in olive oil, or 0.1-percent methylcholanthrene plus 1-percent croton oil in olive oil were inserted into the vaginae of intact strain BALB/c female mice,[6] 60 to 77 days old, three times weekly for the first 30 weeks, then twice weekly for the duration of the experiment (table 1). Although applicators were applied briefly, plugs were permitted to remain in the vaginae until the following day, when they were removed.

The mice were divided into 5 groups and were treated as follows: group 1—olive oil; group 2—croton oil in olive oil; group 3—methylcholanthrene in olive oil once, and 3 days later, continuous treatment with croton oil in olive oil; group 4—methylcholanthrene in olive oil; and group 5—methylcholanthrene plus croton oil in olive oil. The solutions con-

[1] Received for publication December 2, 1954.

[2] Supported by grant DRIR-33 from the Damon Runyon Memorial Fund and grant C-1709 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

[3] This investigation was completed, in part, at the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

[4] With the technical assistance of Dorothy Tabor and Virginia Paul.

[5] Oil Croton, N. F. VII, obtained from Fisher Scientific Co., Elmer and Amend, New York, N. Y.

[6] The author wishes to thank Dr. H. B. Andervont, from whose colonies the mice were derived.

TABLE 1.—*Influence of croton oil, olive oil, and methylcholanthrene on the vaginal epithelium of strain BALB/c mice*

Group	Total* mice (*number*)	Treatment†	Vaginal epithelium									Observation period, all mice (*avg. days*)
			Hyperplasia incidence (*percent*)	Average cell layers, all mice (*number*)	Downgrowths, grade					Acanthosis, incidence (*percent*)	Hyperkeratosis, incidence (*percent*)	
					0 (*number*)	1 (*number*)	2 (*number*)	3 (*number*)	4 (tumors) (*no./%*)			
1	15	O	0	4.5	9	4	2	0	0/0	0	0	410
2	18	C/O	5.5	5.6	10	5	3	0	0/0	5.5	0	392
3	25	M/O—1 ×; C/O	4.0	5.9	10	10	5	0	0/0	4.0	0	401
4	26	M/O	23.1	11.6	2	4	14	4	2/7.7	23.1	15.4	373
5	21	M/C/O	85.7	23.9	0	2	4	8	7/33.3	85.7	57.1	334

*Survivors at time first tumor was observed.

†O=olive oil; C=1% croton oil; M=0.1% methylcholanthrene. All solutions containing methylcholanthrene (groups 3-5) were administered by cotton plug, while olive oil, and croton oil in olive oil (groups 1-3) were administered by applicator.

taining methylcholanthrene were applied by cotton plugs (groups 3–5), while those containing croton oil and olive oil, or olive oil alone were applied with applicators (groups 1–3). Whenever the general condition of an animal necessitated an interruption of treatment, this was subsequently resumed following a suitable period of recovery, or the animal was sacrificed. The mice were examined for tumors 2 months from the start of treatment and every other week thereafter. At autopsy, the vaginae were excised and fixed in Tellyesniczky's fluid. Each vagina was divided arbitrarily into 3 approximately equal parts as follows: *a*) the upper vagina and cervical area, *b*) the mid-portion, and *c*) the lower end, including the vaginal orifice. Sections were cut from each of these areas and stained with hematoxylin and eosin. Some of the specimens were sectioned semiserially and every 4th to 6th section was mounted for study.

Vaginal downgrowths were classified using a modification of the system of grading employed by Suntzeff *et al.* (*1*). The following is a description of the grades used in the present study (table 1): grade 0—no epithelial processes or occasional short ones (figs. 4–6); grade 1—epithelial downgrowths projecting "a moderate distance" into the submucosa; grade 2—processes penetrating "to a considerable depth" and with some branching (figs. 7 and 8); grade 3—deep downgrowths with irregular penetration of connective tissue, appreciable branching of processes, pearl formation—conditions suggestive of precancerous lesions (fig. 7); grade 4—tumors, including papillomas and squamous-cell carcinomas (figs. 1–3). All vaginal tumors were diagnosed following microscopic examination.[7]

Since the thickness of the vaginal epithelium varies considerably during each estrous cycle and may contain 10 to 13 cell layers at estrus (fig. 4), no vagina was considered hyperplastic in the present experiment unless the number of layers exceeded the latter figure. In many instances, *e.g.*, group 5, hyperplastic mucosae contained over 20 cell layers. It has been observed in this laboratory[8] that the vaginae of untreated BALB/c mice of an age comparable to those employed in the present study contain at estrus an average of 4 to 6 layers of spinous cells. Vaginae were considered acanthotic, therefore, when the number of spinous cell layers exceeded 6. Frequently 13 such layers were observed, especially among those vaginae exposed to methylcholanthrene and croton oil. Thus cognizance was taken of, and allowances made for, the cyclic variations normal to the vagina, the conditions reported being appreciably greater than those observed for intact untreated females (*5*). The age of the host also was considered, inasmuch as epithelial downgrowths in the vagina are known to increase with age (*1*).

Results and Discussion

Continued exposure to olive oil, or croton oil in olive oil, had little or no effect on the vaginal epithelium when measured in terms of hyper-

[7] The author is indebted to Dr. Thelma B. Dunn, of the Laboratory of Pathology, National Cancer Institute, for her assistance.

[8] Unpublished data.

plasia, vaginal downgrowth, acanthosis, and hyperkeratosis (figs. 5, 6—groups 1, 2; table 1). The lining of the vaginal mucosa in some of the mice treated with olive oil alone and croton oil in olive oil (groups 1, 2) consisted in part of squamous to cuboidal cells with pyknotic nuclei and dark-staining cytoplasm. Other vaginal mucosae from the same groups contained surface cells that were polyhedral or cuboidal to low columnar and in which the cytoplasm stained lightly with eosin. The nuclei in the latter cells were oval to spherical in shape and stained well with hematoxylin. Evidence of keratinization was lacking.

The results with croton oil on vaginal epithelium (table 1) are in marked contrast with those reported for the skin, since numerous alterations are known to occur in the latter organ following prolonged treatment with this substance (*6*, *7*). It may be that the absence of hair follicles and sebaceous glands, the presence of mucus, and the cyclic changes accompanying estrus were factors which contributed to this difference. Levy *et al.* (*8*) observed marked changes in the skin following exposure to 9,10-dimethyl-1,2-benzanthracene but obtained none when the mucosa of the lip was similarly exposed. It was suggested that these results might be due to the protective action of mucus or to the absence of sebaceous glands in the lip. On the other hand, Stewart (*9*) has questioned the necessity of hair follicles and sebaceous glands for the development of skin tumors in view of the induction of tumors of the esophagus and forestomach of mice with the same carcinogens employed for the skin.

The changes produced in the vagina following exposure to methylcholanthrene once and then continuously to croton oil (group 3) did not differ from those observed following treatment with olive oil or with 1-percent croton oil in olive oil (table 1). Pathologic alterations including squamous-cell carcinomas were produced, however, when the solution of methylcholanthrene was applied to the vagina repeatedly (group 4). Spontaneous tumors of the vagina have rarely been observed in the mouse (*10*, *11*). Although few or no vaginal tumors have been reported in the experiments of some investigators following exposure of vaginal epithelium to tar or the hydrocarbon carcinogens (*12*, *13*), tumors of this type were induced with these carcinogens in the experiments of others (*14–16*). In the present experiment, 2 of the mice subjected to methylcholanthrene in olive oil bore squamous-cell carcinomas. Histologic examination revealed numerous alterations in the vaginal mucosa, as summarized in group 4, table 1. Thus vaginal hyperplasia was increased and the depth of penetration and the character of the epithelial processes were altered in the direction of neoplasia. The incidence of acanthosis and hyperkeratosis was similarly increased. Stewart (*9*) has indicated that the latter two conditions appear to be associated with an acceleration in forestomach tumorigenesis in mice. Many more pathologic alterations and more vaginal tumors were produced in the mice that received croton oil and carcinogen (group 5) than in those given carcinogen alone (group 4), despite a shorter period of treatment for the former mice (figs. 1–3, 7, 8). The incidence of vaginal hyperplasia and acanthosis increased from 23.1

to 85.7 percent, and the number of hyperkeratotic vaginae rose from 15.4 to 57.1 percent (table 1). Five of the mice in group 5 bore squamous-cell carcinomas and an additional 2 bore vaginal papillomas The first tumor was discovered 87 days from the start of the experiment, as compared to 227 days for the first tumor in group 4. Two of the mice were observed with small vaginal lesions which resembled early squamous-cell carcinomas. Since an unqualified diagnosis could not be made, however, the lesions were classified as grade 3, rather than grade 4 (group 5, table 1). Comparison of tumor incidences for groups 4 and 5 gave a χ^2 value of 3.5, which approaches significance at the 0.05 level.

It is obviously not possible to conclude from these data alone that croton oil enhanced the action of methylcholanthrene on the vaginal epithelium. Such a conclusion is warranted, however, when to these results are added those on vaginal hyperplasia, epithelial downgrowth, acanthosis, and hyperkeratosis. The fact that croton oil alone appeared to have no effect in group 2 but a considerable effect when applied together with methylcholanthrene in group 5 suggests that one or more of the following may have played a part: *a*) vaginal epithelium was exposed to croton oil for a longer period of time in group 5 than in group 2 following each application of solution (plug *vs.* applicator mode of administration); *b*) croton oil may have acted in conjunction with methylcholanthrene; or *c*) the vaginal epithelium may have been altered by the carcinogen, thus making the tissue more susceptible to the action of croton oil. The stimulating influence observed with croton oil may not be related to the promoting action of this substance in skin tumorigenesis. Since both methylcholanthrene and croton oil were applied together in the present study, the possibility of a solvent effect cannot be eliminated. Also, the stimulation observed with croton oil may be a reflection of its irritant and not its promoting action. It is well known in the case of skin tumorigenesis that the cocarcinogenic action of croton oil is not explainable on the basis of its irritant or hyperplastic action (*17*).

The papillomas and carcinomas induced in the present investigation (figs. 1–3) arose near the middle and upper portions of the vagina but did not extend to the cervix. The papillomas were generally broad-based and contained numerous mitotic figures. The tumors were frequently covered with many layers of keratin, while keratinized epithelial cysts were commonly observed. The nuclei, especially of the basal and spinous cells, were hyperchromatic and displayed irregularities in size and shape. Cellular transformation was suggested by the presence of spindle-shaped cells at the base of some of the tumors. The nuclear and cellular changes described also were present and, to a greater extent, in the carcinomas than in the papillomas; the carcinomas were large growths which penetrated to a considerable depth into the vaginal wall. Frequently, epithelial strands infiltrated the muscle tissue and extended to the serosa. The stroma, characterized generally by edema and inflammation, was scanty and was infiltrated by round cells. Dilatation of blood vessels was frequently observed.

Fishman *et al.* (*14*) obtained a much greater yield of vaginal tumors in strain A mice than was observed with strain BALB/c mice in the present experiment. This increase in tumor incidence may have been due to the use by these authors of 5-percent benzpyrene-cholesterol pellets instead of the 0.1-percent methylcholanthrene solution employed in the present experiment. Also, strain A may be more susceptible than strain BALB/c to the induction of vaginal tumors.

Summary

The vaginae of intact strain BALB/c mice were exposed repeatedly to solutions of croton oil, methylcholanthrene, or methylcholanthrene and croton oil, all in olive oil, or to olive oil alone. Exposure to methylcholanthrene in olive oil resulted in considerable epithelial downgrowth and branching, hyperplasia, acanthosis, and hyperkeratosis. In addition, squamous-cell carcinomas were induced. Croton oil enhanced the action of methylcholanthrene. Treatment of the vaginal epithelium with methylcholanthrene and croton oil in olive oil produced an intensification of epithelial downgrowth and more extensive branching in the submucosa, a higher incidence of hyperplasia, acanthosis, and hyperkeratosis, and an increase in tumor incidence. These alterations in the vagina were not observed following treatment with the croton oil–olive oil solution, with olive oil alone, or after treatment once with the methylcholanthrene and olive-oil solution and then continuously with croton oil in olive oil.

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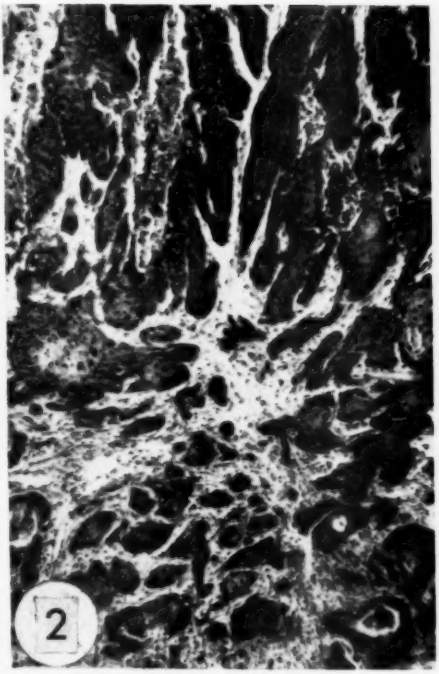
PLATE 16

FIGURE 1.—Section of a vagina bearing papillomas induced by repeated exposure to a solution containing methylcholanthrene, croton oil, and olive oil. Hematoxylin and eosin. × 75

FIGURE 2.—Squamous-cell carcinoma of the vagina induced by repeated exposure to a solution containing methylcholanthrene, croton oil, and olive oil. Hematoxylin and eosin. × 75

FIGURE 3.—A portion of figure 2, *lower left corner*, at a higher magnification. Some of the carcinomatous cells in this section appear fusiform. Hematoxylin and eosin. × 205

FIGURE 4.—Cross section of the vagina of an adult untreated BALB/c mouse in estrus. Hematoxylin and eosin. × 205



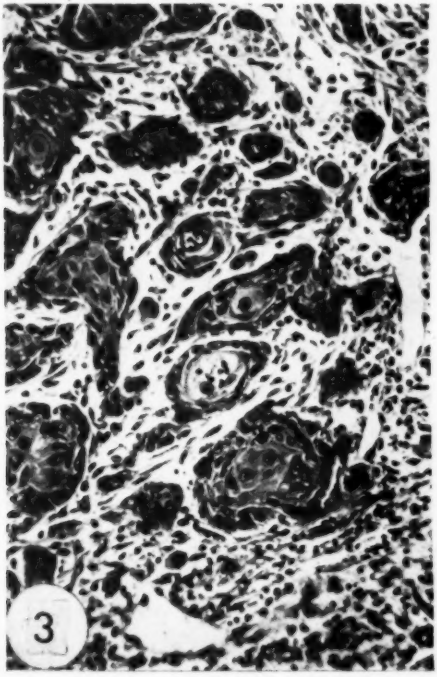


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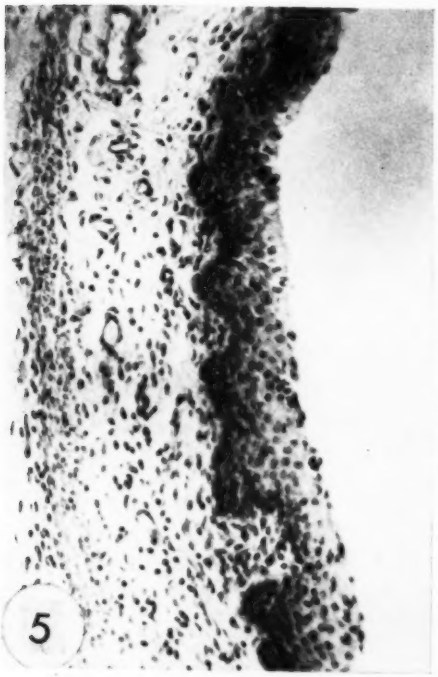
PLATE 17

FIGURE 5.—Cross section of the vagina of a mouse which was exposed repeatedly to a solution containing croton oil and olive oil over a period of 394 days. Hematoxylin and eosin. × 205

FIGURE 6.—Cross section of the vagina of a mouse which was exposed repeatedly to olive oil over a period of 495 days. Hematoxylin and eosin. × 205

FIGURE 7.—Cross section of the vagina of a mouse exposed repeatedly to a solution containing methylcholanthrene, croton oil, and olive oil over a period of 243 days. Note hyperkeratosis, cyst formation, hyperplasia, and excessive downgrowth and folding of mucosa. Region adjacent to arrow on *left* illustrates a grade 2 lesion; that adjacent to *right* arrow, a grade 3 lesion. Hematoxylin and eosin. × 14

FIGURE 8.—Lower central portion of figure 7 at higher magnification. Hematoxylin and eosin. × 75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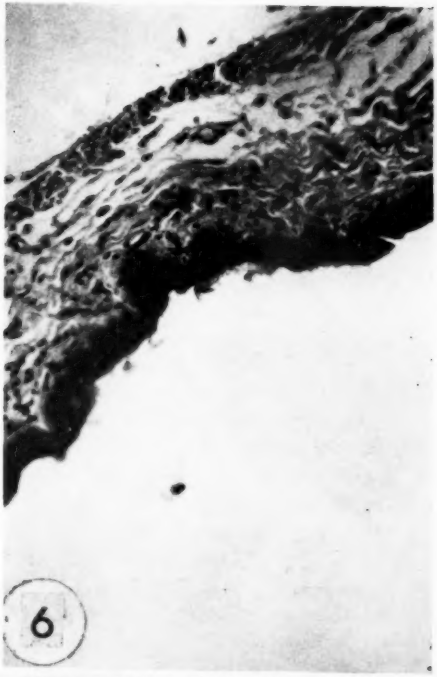
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Trend of Lung-Cancer Mortality in the United States: Some Limitations of Available Statistics [1]

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Increasing interest in the trend of lung-cancer mortality prompted this analysis of United States mortality statistics related to cancer of the respiratory system. Emphasis will be placed on problems arising out of incompleteness of geographic coverage prior to 1933, and lack of uniformity in methods of classifying cause-of-death information derived from death certificates. Estimates will be presented of the trend of reported mortality from cancer of the lung and of the larynx in the United States from 1914 to 1950, which attempt to take into account changes in methods of death classification. These estimates will be compared with estimates obtained by conventional methods. This comparison will help clarify the question of whether the many changes in cause-of-death classification procedures have seriously impaired comparability of lung-cancer mortality statistics.

Factors relevant to validity of cause-of-death information on death certificates, such as improvement in methods of diagnosis, have undoubtedly affected to a significant degree the trend of recorded mortality from cancer of the respiratory system. Such factors, however, are generally not subject to measurement and are therefore beyond the scope of this report.

A. The United States Death Registration Area Prior to 1933

In any study of United States mortality experience, it is necessary to remember that annual mortality data for the entire United States are not available for years prior to 1933. The Death Registration Area was established in 1880. Requirements for admission were at least 90-percent completeness of death registration and evidence that deaths were recorded properly under adequate registration laws. Collection of annual vital statistics based on information recorded on death certificates was begun in 1900. The Death Registration Area consisted of Death Registration States and Death Registration Cities in nonregistration states. Its growth from 1905 to 1933 is illustrated in table 1. The population of

[1] Received for publication January 11, 1955.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

Death Registration Cities in nonregistration states was about 7 percent of the total Death Registration Area population from 1914 to 1917 and between 1 and 2 percent from 1918 to 1932. All the subsequent discussion and analysis of mortality statistics for years prior to 1933 will be limited to the Death Registration States.

TABLE 1.—*Growth of the Death Registration Area, 1905–1933*

Year	Death registration area in continental U. S.		
	Percent of continental U. S. population	Number of Death Registration States*	Number of Death Registration Cities in nonregistration states
1933	100	49	0
1925	90	41	24
1915	68	25	140
1905	40	11	123

*States, including the District of Columbia, completely included in the Death Registration Area.

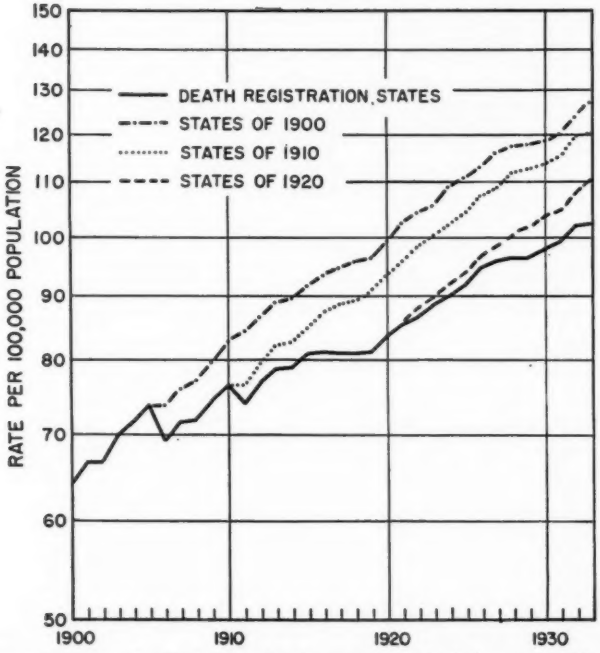
Prior to 1914, the Death Registration States comprised less than two thirds (prior to 1908, less than a half) of the population of continental United States.[3] Moreover, its expansion was not a process of uninterrupted growth. States were dropped and readmitted on several occasions. With the exception of the District of Columbia,[4] all the Registration States of 1905 were east of Illinois and north of the Ohio River and the Mason-Dixon line. In 1909, all the 12 states in this region, but only 7 states in other parts of the country, were Registration States. As recently as 1921, only 23 of the 37 states outside the northeastern region were among the Registration States.

Mortality rates for specified causes of death in any given state often differ widely from corresponding rates in other states. In general, crude mortality rates for cancer of all sites is highest in states that were admitted to the Death Registration Area early in its development. This is clearly illustrated in text-figure 1, which shows crude cancer-mortality rates for selected groups of states from 1900 to 1933. The Death Registration States of 1900 consistently experienced the highest rates, followed in descending order by the Death Registration States of 1910, those of 1920, and finally (in 1933) the entire area of continental United States (*1*). Lung-cancer mortality probably followed a similar pattern but exactly comparable data are not available.

Hoffman and Gilliam (*2*) studied the geographic distribution of mortality attributed to lung cancer in 1948 and 1949, taking into account the age, race, sex, and residence (urban-rural) composition of each state's population. They found that, in comparison with the total United States experience, 8 states had significantly higher and 19 states had significantly lower standardized lung-cancer mortality rates ($P<0.01$).

The earliest published mortality data for cancer of the respiratory system in the Death Registration States are for the year 1914. Table 2 shows crude and standardized lung-cancer mortality ratios for the period

[3] The proportion of total land area included was less than two thirds prior to 1923 and less than half prior to 1918.
[4] Throughout this discussion the District of Columbia will be considered as a state.



TEXT-FIGURE 1.—Crude death rates for cancer: Selected areas, 1900–1933.

1948 to 1949, of the Death Registration States of 1914 and of the nonregistration states of 1914. The differences between the crude mortality ratios as well as those between the standardized mortality ratios are statistically significant ($P<0.0001$). The difference between the two standardized ratios is much smaller than the difference between the two crude ratios. Thus in 1948 to 1949 some, but not all, the differences in recorded lung-cancer mortality in the Registration States of 1914 as compared with nonregistration states of 1914 may be accounted for on the basis of differing age, race, sex, and residence (urban-rural) characteristics of the two areas.

TABLE 2.—*Crude and standardized mortality ratios for lung cancer, 1948–1949: Death Registration States of 1914 and nonregistration states of 1914*

Geographic area	Lung-cancer mortality ratios, 1948–1949* (U. S. = 100)	
	Crude	Standardized†
Registration States of 1914	114	104
Nonregistration states of 1914	77	90
P values for differences between the geographic areas	<0. 0001	<0. 0001

*Based on observed and expected numbers of deaths reported by Hoffman and Gilliam (2), table 3.
†Standardized for age, race, sex, and residence (urban-rural).

Without further belaboring the point, it is clear that geographically comparable mortality data for the entire United States prior to 1933 do not exist. States admitted to the Death Registration Area early in its development represent a highly biased sample of the United States. Their recorded specific mortality experience may be regarded as the best available index to corresponding rates for the entire United States but must be interpreted with caution. The later admission of states with low rates (even when age-adjusted) has the effect of lowering the trend of recorded lung-cancer mortality in the expanding Death Registration Area.

B. Classification of Cause-of-Death From Medical Information on Death Certificates

Another major problem in studying mortality trends arises from periodic changes in methods of classifying cause-of-death information derived from death certificates. In general, the United States has used the decennial revisions of the *Manual of the International List of Causes of Death* (3–7) for death classification. Comparability of rubrics is sometimes lost in the process of revision. That this occurred in the case of cancer of the respiratory system is clearly shown in table 3.

TABLE 3.—*Statistical classification of deaths ascribed to cancer of the respiratory system: Titles and terms used in each Decennial Revision of the International List of Causes of Death, as adopted for use in the United States, since 1914*

2nd Revision, 1909 used 1914–1920

45 Cancer and other malignant tumors of other organs or of organs not specified
- A. Larynx
  - *Cancer of larynx*
- B. Lung and pleura
  - *Cancer of:*
    - *anterior mediastinum*
    - *lung*
    - *mediastinal gland*
    - *mediastinum*
    - *pleura*
  - *Pulmonary cancer*
- Seven other subtitles (not related to the respiratory system)
- X. Others of this class
  - Includes 90 terms, of which only the following pertain to the respiratory system:
  - *Cancer of:*
    - *accessory sinus*
    - *antrum*
    - *bronchi*
    - *posterior nares*
    - *trachea*

Note: The 2d revision was in use from 1910 to 1920 but the subtitles (A, B, etc.) shown above were first used in 1914. At the request of the American Society for the Control of Cancer, the U. S. Bureau of the Census subdivided the 7 International List titles covering the subject of cancer under 29 headings. Title 45 includes 127 terms, of which only 12 pertain to the respiratory system (7). For years prior to 1914, it is impossible to obtain mortality data for the Death Registration Area related exclusively to cancer of the respiratory system.

3rd Revision, 1920 used 1921–1929

49 Cancer and other malignant tumors of other or unspecified organs
- A. Larynx
- B. Lung and pleura
- Seven (1921–1924) or 8 (1925–1929) other subtitles (not related to the respiratory system)
- X. Others of this class

Note: A list of terms included under the above subtitles was not published. However, titles 49A, 49B, and 49X probably correspond very closely to titles 45A, 45B, and 45X as used during the years 1914–1920. The International List title 49 includes 136 terms, of which only 16 pertain to the respiratory system, and has no subtitles (5). By means of these subtitles, the U. S. Bureau of the Census provided more detailed classification than required by the International List.

4th Revision, 1929 used 1930–1938

47 Cancer and other malignant tumors of the respiratory system
- A. Larynx
  - *Cancer of:*
    - *epiglottis*
    - *glottis*
    - *larynx*
- B. Lungs and pleura
  - *Cancer of:*
    - *anterior mediastinum*
    - *lung*
    - *mediastinal gland*
    - *mediastinum*
    - *pleura*
  - *Pulmonary cancer*
- C. Other respiratory organs
  - *Cancer of:*
    - *bronchi*
    - *nasal cavity*
    - *nasopharynx*
    - *nostril*
    - *posterior nares*
    - *trachea*

TABLE 3. (Cont.)—*Statistical classification of deaths ascribed to cancer of the respiratory system: Titles and terms used in each Decennial Revision of the International List of Causes of Death, as adopted for use in the United States, since 1914*

53 Cancer and other malignant tumors of other or unspecified organs
A, B, C, E (not related to the respiratory system)
D. Bones (except jaw)
Includes 16 terms, of which only the following pertain to the respiratory system:
Cancer of:
accessory sinus
antrum
antrum of Highmore
frontal sinus
maxillary sinus

Note: International List titles 47 and 53 do not contain subtitles. Terms included under each subtitle, as used by the U. S. Bureau of the Census, have not previously been published. They were provided by the Mortality Analysis Branch of the National Office of Vital Statistics. The terms *nostril* and *posterior nares* are included under title 53 of the International List but under subtitle 47C of the U. S. Bureau of the Census.

5th Revision, 1938 used 1939–1948

47 Cancer of the respiratory system
(a) Larynx
Cancer of:
epiglottis
glottis
larynx
(b) Trachea
Cancer of Trachea
(c) Bronchus
Bronchogenic carcinoma
Cancer of bronchus
(d) Lung
Cancer of lung
Pulmonary cancer
(e) Pleura
Cancer of pleura
(f) Mediastinum and unspecified sites
Cancer of:
mediastinal gland
mediastinum
respiratory system
(part not specified)

55 Cancer of other and unspecified organs
(a), (b), (c) (not related to the respiratory system)
(d) Nasal cavity and accessory sinuses
Cancer of:
accessory sinuses
antrum
antrum of Highmore
frontal sinus
maxillary sinus
nasal cavity
(e) Other and unspecified organs
Includes 27 terms, of which only the following could possibly pertain to the respiratory system:
Cancer of:
chest
thoracic cavity
thorax

6th Revision, 1948 used 1949–

160 Malignant neoplasm of nose, nasal cavities, middle ear, and accessory sinuses
Malignant neoplasm of:
accessory sinus (any)
Eustachian tube
frontal sinus
maxillary sinus
middle ear
nasal cavity
nasal septum
nose, NOS
nose, internal

161 Malignant neoplasm of larynx
Cancer of cricoid cartilage
Malignant neoplasm of:
arytenoids
epiglottis
glottis
larynx
vocal cords (*false*)

162 Malignant neoplasm of trachea, and of bronchus and lung specified as primary
Bronchogenic carcinoma
Malignant neoplasm (of):
bronchus, *lung*, *pleura*, *pulmonary* } specified as primary site
trachea, NOS, or primary
Pancoast's tumour unless specified as benign

163 Malignant neoplasm of lung and bronchus, unspecified as to whether primary or secondary
Malignant neoplasm (of):
bronchus, *lung*, *pleura*, *pulmonary* } unspecified as to whether primary or secondary

164 Malignant neoplasm of mediastinum
Malignant neoplasm of:
mediastinum
thoracic organs, unspecified

165 Malignant neoplasm of thoracic organs (secondary)
Malignant neoplasm of:
bronchus, *lung*, *mediastinum*, *pleura*, *thorax*, *trachea* } specified as secondary
respiratory organs NOS
This title is not to be used for primary death classification if the site of the primary neoplasm is known.

Note: "NOS" is an abbreviation for "not otherwise specified."

Table 3 gives all the titles and terms relating to cancer of the respiratory system appearing since 1914 in tabular lists of each decennial revision of the *Manual of the International List of Causes of Death*, as adopted for use in the United States. Diagnostic terms included under each title or subtitle are shown in italics. The U. S. Bureau of the Census should be commended for its early recognition of the need for detailed classification of cancer deaths. From 1914 to 1938 the Bureau supplemented the International List by means of additional subtitles (A, B, etc., in table 3). Except for this, there would be no respiratory system cancer-mortality statistics for years prior to 1930. From 1930 to 1938, less detailed information would have been available. Mortality statistics (*8–11*) are based on the total number of deaths classified under each title and subtitle. The number of deaths ascribed to any of the inclusion terms (shown in italics in table 3) is not available.

Inspection of table 3 shows that, with the single exception of the larynx, strictly comparable mortality statistics for cancer of the respiratory system or any of its components are unobtainable for the entire period 1914 to 1950. It is impossible to obtain the number of deaths attributed to cancer of the bronchus—bronchogenic carcinoma—prior to 1939. Prior to 1930, it is not even possible to obtain the total of deaths from bronchogenic carcinoma plus any combination of respiratory system cancers. This is a point that has often been overlooked in discussions of lung-cancer mortality trends. The exclusion of bronchogenic carcinoma results in an understatement of "lung-cancer" deaths. This in turn artificially magnifies the apparent recorded increase in lung-cancer mortality for 1939 and all later years as compared with years prior to 1939.

It is of interest to note the gradually increasing preference of physicians for the terms *bronchogenic carcinoma* or *cancer of the bronchus* over the terms *pulmonary cancer* or *cancer of the lung* as diagnoses recorded on death certificates. The number of deaths that physicians actually attributed to the former causes prior to 1939 is unknown. In 1930 it could not have been more than 211, which is only 7.4 percent of all deaths ascribed to respiratory cancer other than larynx (table 4). It was undoubtedly substantially fewer than 211 because this figure, in addition to cancer of the bronchus, includes five nonpulmonary sites of respiratory system cancers (table 3). From 1939 to 1948 deaths attributed by physicians to cancer of the bronchus (or bronchogenic carcinoma) were classified in a subtitle that included no other cancers. Table 4 shows that during this period recorded deaths from cancer of the bronchus (title 47c) increased from 20.8 to 34.1 percent of all deaths ascribed to cancer of the respiratory system other than larynx (titles 47b-f). During this decade the crude death rate attributed to cancer of the bronchus increased 3.4 times while that for cancer of the lung (title 47d) increased only 1.8 times. Though table 4 does not show it, more detailed examination of the data available suggests that the term *bronchogenic carcinoma* assumed real popularity among physicians as a stated cause of death in the latter part of the decade 1939 to 1948. The evidence seems clear that prior

to that time the physician was more likely to choose the term *cancer of the lung* for what was with increasing frequency later called *bronchogenic carcinoma*. Since 1949 both these terms have been statistically classified in such a manner that separate totals cannot be obtained (table 3).

TABLE 4.—*Number of deaths attributed to cancer of the respiratory system other than larynx and classified under specified subtitles of the International List during first and last years of use of fourth and fifth revisions: Death Registration States, 1930, and continental United States, 1938, 1939, and 1948*

Year	Number of deaths classified under specified subtitles (other than larynx) of cancer of the respiratory system*		Percent of deaths from cancer of respiratory system (other than larynx) ascribed to "other respiratory organs"† (1930–1938) or to "bronchus" (1939–1948)
	47 C, Other respiratory organs† (1930–1938) or 47 (c), bronchus (1939–1948)	47 B, Lungs and pleura (1930–1938) or 47 (b), (d), (e), (f), trachea, lung, pleura, and mediastinum (1939–1948)	
	(1)	(2)	$\frac{(1)}{(1)+(2)} \times 100$
1930	211	2, 626	7. 4
1938	1, 481	5, 300	21. 8
1939	1, 516	5, 763	20. 8
1948	5, 838	11, 264	34. 1

*See table 3 for complete definitions of these subtitles.
†Includes "bronchus."

Classification of deaths attributed to cancer of the trachea presents problems almost identical with those described for bronchogenic carcinoma. However, the number of deaths involved is an insignificant proportion of the number of deaths attributed to cancer of the respiratory system—0.8 percent in 1939 and 0.4 percent in 1948.

Mortality statistics that may be considered representative of substantially the entire respiratory system as a unit did not become available until 1939. However, deficiencies in this respect during the years 1930 to 1938 (due to lack of data on numbers of deaths ascribed to cancer of the sinuses) are not very large—probably less than 5 percent.

Contrary to the current and all previous revisions of the *Manual of the International List of Causes of Death*, cancer of the nasopharynx was included with cancer of the respiratory system from 1930 to 1938. Only in the sixth revision are cancers of the Eustachian tube and middle ear included under cancers of the respiratory system. Other inconsistencies that disrupt comparability of titles could be cited.

The sixth revision of the International List is the first in which cancers of some sites are classified under two or three different titles. In such cases, the title designation depends upon whether the cancer is 1) specified as primary site; 2) unspecified as to whether primary or secondary; or 3) specified as secondary. This trichotomy is not applied uniformly to all parts of the respiratory system. It is not used at all with reference to the nose, nasal cavities, middle ear, accessory sinuses, or larynx; only

partially used for the trachea and mediastinum; but consistently used for the bronchus, lung, and pleura. The grouping of terms under the six titles embracing cancer of the respiratory system is such that none of these titles represents only cancers that are specified as primary. However, at least one study (*12*) suggests that a clinical diagnosis of "lung cancer," unspecified as to whether primary or secondary, almost always indicates a primary lung-cancer case for which the reporting physician simply omitted the word "primary."

Prior to 1949, editions of the *Manual of Joint Causes of Death* (*6, 13–15*) clearly set forth which of two or more diagnoses appearing on a death certificate was to be selected as the underlying (primary) cause of death. The first of these, published in 1914, states that as a general rule cancer is to be coded as the cause of death when reported in conjunction with other diseases, but if "the jointly reported cause is a very rare and fatal disease—*e.g.*, bubonic plague, Asiatic cholera, etc.—the latter should be preferred." The second edition, 1925, follows the same principle but gives considerably more detail. Cancer of other or unspecified organs (including lung and larynx) is preferred over all causes of death except typhus, cholera, plague, yellow fever, leprosy, glanders, anthrax, rabies, tetanus, other epidemic diseases, accidents, external violence, and cancer of the buccal cavity, digestive system (except liver and biliary passages), female genital organs, breast, and skin. For cancer of the respiratory system the third (1933) and fourth (1939) editions differ from the second only in that cancer of the skin, if listed as a joint cause, no longer takes precedence over cancer of the respiratory system as the coded cause of death. Prior to 1949, when cancers of two or more sites were mentioned on a death certificate, the primary site was only infrequently specified. This gave rise to a difficult problem of selection. The site selected for tabulation was generally the presumptive primary site (according to the rules summarized above) except when such a site was specified as secondary. Statistical classification of cancer of the respiratory system as an underlying cause of death thus depended on predetermined rules as well as on the accuracy with which physicians reported cancer on death certificates. Since 1949 the underlying cause of death has been designated in general by the certifying medical practitioner.

The most recent national statistics on associated causes of death in relation to underlying causes are for the year 1940 (*16*). Fifty-five percent of all death certificates showed more than one cause of death. Among the 9,543 deaths ascribed to title 47, "Cancer of the respiratory system," there were 2,410 in which cancer of another site was mentioned as an associated cause. Only 39 of the latter were cancer of the skin. Prior to 1933, the underlying cause of death in such instances would have been coded as cancer of the skin. In 7,375 other death certificates the diagnosis of a cancer of the respiratory system was mentioned. The underlying cause of death was coded as cancer (other than under title 47) in 7,321 of these cases and as noncancerous conditions in the remaining 54 cases.

For death certificates with two or more causes of death, rules for selection of the underlying cause were fairly constant from 1914 to 1948. The effect of the currently used death classification procedures, adopted for use in 1949, upon comparability of mortality statistics for earlier years has been comprehensively studied (*17*). Though undoubtedly influencing the recorded trend of lung-cancer mortality, the problem of the statistical selection of the underlying cause of death does not appear to be an important source of error. Comparability ratios, taking into account this and certain other problems, are available for the adjustment of reported lung-cancer mortality rates.

For some purposes it is desirable to classify lung cancers according to their microscopic pathology into groups such as squamous-cell carcinoma, adenocarcinoma, and undifferentiated carcinoma. Such information is not available from mortality statistics that are based on death certificates.

C. Trend of Recorded Mortality From Cancer of the Respiratory System in the United States, 1914 to 1950

In the light of the preceding discussion and table 3, one might select certain titles from each revision of the International List to serve as the basis for a study of mortality trends. The necessity for standardization of rates for demographic factors, such as age, race, and sex, places further limitations on the choice of rubrics. While the total number of deaths ascribed to causes under each title is available annually for the Death Registration Area as a whole, mortality data by age, race, and sex have been available only for certain titles or groups of titles.

Accordingly, the titles listed in table 5 were selected as the basis for estimating lung-cancer mortality trends for the years 1914–1950. For convenience, these titles will hereafter be referred to as "lung cancer." Various different groups of titles have been used in other reports. If the years prior to 1930 were excluded from consideration, it would be possible to select more appropriate rubrics. As a group, the titles selected could be more accurately described as "cancer of the respiratory system other than larynx" than as "lung cancer." Even this designation is not entirely correct. In 1950, the number of deaths coded as due to cancer of the nose, nasal cavities, middle ear, and accessory sinuses (title 160) was 3.3 percent of the number coded to "lung cancer" (titles 160, 162–164). Deaths ascribed to secondary cancer of thoracic organs (title 165) could not be included because of lack of mortality data by age, sex, and race. For the same reason, such deaths could not be excluded from the data for years prior to 1949 However, in 1950 the number of death s coded under title 165 was only 1.5 percent of the number coded to titles 160, 162–164. Errors referable to inclusion of title 160 and exclusion of title 165 are in opposite directions. The combined effect of these and other sources of incomparability can be at least partly overcome by means of comparability ratios.

Table 5 also indicates, for selected titles of the second through fifth revisions of the International List, major deficiencies in comparability

TABLE 5.—*International List titles used for estimation of mortality from cancer of the respiratory system other than larynx, comparability of titles, and comparability ratios: 1914–1950*

Years	*International List*		Principal deficiencies in comparability with 6th revision titles		Comparability ratio*
	Revision number	Title numbers	Additions	Deletions	
1949–50	6th	160, 162–164	—	—	—
1939–48	5th	47 b–f	Includes title 165 (thoracic organs, secondary) except thorax.	Excludes title 160 (nose, nasal cavities, middle ear, and accessory sinuses).	0. 946†
1930–38	4th	47 B, C	Includes nasopharynx and title 165 except thorax.	Excludes title 160 except nose and nasal cavities.	. 926‡
1921–29	3rd	49 B	Includes title 165 except bronchus, thorax, and trachea.	Excludes bronchus, trachea, and title 160.	1. 000§
1914–20	2nd	45 B	Same as 3rd revision.	Same as 3rd revision.	1. 000

*Factor to be applied to number of recorded deaths to obtain number of deaths comparable to 6th revision titles 160, 162–164.

†Based on a study of comparability by National Office of Vital Statistics (*17*).

‡Comparability ratio of 4th to 5th revision titles, based on report of Dunn and Shackley (*18*), is 0.979. Product of this ratio and ratio of 5th to 6th revision titles is taken as the comparability ratio for 4th to 6th revision titles.

§Principal difference between 3rd and 4th revisions is inclusion of bronchi, nasal cavity, nasopharynx, and trachea under a new subtitle, 47 C, of the 4th revision. In 1930, there were 2,626 deaths ascribed to 47 B and 211 to 47 C in the Death Registration States. The comparability ratio of 3rd to 4th revisions is taken as $\frac{2626+211}{2626}$, or 1.080. Product of latter ratio and ratio for 4th to 6th revisions is taken as comparability ratio for 3rd to 6th revision titles.

with selected sixth revision titles. Comparability ratios shown in the last column of table 5 are correction factors computed in an effort to achieve comparability of mortality data through five revisions of the International List. They do not correct for lowering of the recorded lung-cancer mortality trend in the expanding Death Registration Area which occurred as a result of the later admission of states with generally lower rates. Comparability ratios for some causes of death, including malignant neoplasms, vary considerably with age (*17*). Age-specific comparability ratios for cancer of the lung are not available. While comparability ratios have this and other shortcomings, they were computed for the purpose of evaluating the effect of decennial International List revisions and changes in the *Manual of Joint Causes of Death* on comparability of lung-cancer mortality data. Comparability ratios for the years 1914 to 1948 fluctuate between 1.000 and 0.926. It seems surprising that, in spite of the many sources of incomparability of International List rubrics, the correction factors it was possible to compute do not give rise to an estimated trend of lung-cancer mortality that differs materially from previously reported estimates.

Mortality rates for cancer of the lung and of the larynx, for the total population from 1914 to 1950, are shown in table 6. They are based on deaths reported in the Death Registration States in 1914 and 1930[5] and on deaths reported in continental United States for the years 1935, 1940, 1945, and 1950 (*8–11*). Death rates[6] were standardized by the direct method (*19*) for age, race, and sex. The standard populations used were white males, white females, nonwhite males, and nonwhite females as enumerated by age in the 1950 census (*20*). Each standardized rate was then multiplied by the appropriate comparability ratio. These products are the adjusted standardized rates in table 6.

Use of the age distribution of each of the four race-sex groups in the standard population, rather than a single age distribution for all, yields standardized rates that are more realistic than would otherwise be the case. Comparisons may be made of the *rate* of increase in recorded lung-cancer mortality experienced by each of the four race-sex groups. However, because this method of standardization utilizes different age distributions in the standard population for each of the four race-sex categories, it does not provide an accurate basis for comparisons of the *magnitude* of their individual mortality risks. For this reason rates by race and sex, readily obtainable as by-products of the computation, are not given.

The adjusted standardized mortality rate for lung cancer among all persons increased from 6.6 per million in 1914 to 127.9 per million in 1950. The latter is about 19 times as large as the former. It was shown in

[5] It is unfortunate that from 1915 through 1929 published lung- and larynx-cancer mortality statistics do not show deaths by age, race, and sex—only total numbers of deaths are available. For this reason, table 6 does not contain rates for any year between 1914 and 1930.

[6] Death rates from 1940 to 1950 relate deaths in continental United States to the total population, including armed forces overseas. Since overseas military personnel developing cancer were usually transferred to the continental United States for treatment, for all practical purposes it may be assumed that the death rates in table 6 include their mortality experience.

TABLE 6.—*Number of deaths per 1,000,000 population ascribed to cancer of the lung* and of the larynx: Death Registration States, 1914–1930, and continental United States, 1935–1950*

Site of cancer and kind of mortality rate	Years						Proportional increase in mortality rates	
	1914	1930	1935	1940	1945	1950	Ratio: 1950 rate / 1914 rate	Ratio: 1950 rate / 1930 rate
Lung*								
Crude mortality rate†	5.5	24.2	39.7	61.2	85.6	128.1	23.3	5.3
Standardized mortality rate‡	6.6	28.9	45.1	65.1	88.1	127.9	19.4	4.4
Adjusted standardized mortality rate§	6.6	26.7	41.8	61.6	83.4	127.9	19.4	4.8
Larynx								
Crude mortality rate†	5.2	8.3	9.1	11.0	11.9	12.3	2.4	1.5
Standardized mortality rate‡	7.0	10.3	10.6	11.9	12.4	12.2	1.7	1.2
Adjusted standardized mortality rate ‖	6.8	10.0	10.4	11.6	12.1	12.2	1.8	1.2

*See table 5 for definition of this title.

†Recorded deaths per million persons.

‡Recorded deaths per million persons standardized for age, race, and sex on basis of age distribution of white males, white females, nonwhite males, and nonwhite females as enumerated in the United States census of 1950.

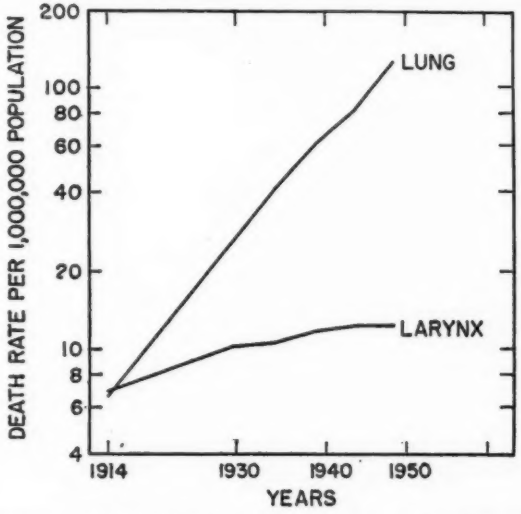
§Standardized mortality rate adjusted for comparability of titles of the various revisions of the *International List of Causes of Death*. See last column of table 5 for comparability ratios.

‖Comparability ratio for 5th to 6th revisions is 0.975 (*17*); for 4th to 5th is 0.999 (*18*), hence for 4th to 6th is 0.975 × 0.999 or 0.974; for 2nd and 3rd to 4th is assumed to be 1.000 (precise data not available), hence for 2nd and 3rd to 6th is taken as 0.974.

table 2 that the Death Registration States of 1914 have in recent years had significantly higher lung-cancer mortality rates than other states. The estimate of a 19-fold increase in the United States from 1914 to 1950 is therefore probably conservative. Between 1930 and 1950 recorded lung-cancer mortality increased 4.8 times.

Comparisons of three kinds of lung-cancer mortality rates—crude, standardized (conventional procedure), and adjusted standardized—may be made by inspection of table 6. Corrections for age, race, and sex (standardized rates minus crude rates expressed as a percentage of crude rates) are positive, ranging from about 20 percent in 1914 and 1930 down to zero [7] in 1950. The corrections applied to obtain comparability of International List rubrics and changes in *Manual of Joint Causes of Death* (the negative complement of the comparability ratios of table 5) fluctuate within the range of zero and minus 7.4 percent. The net effect of both corrections (adjusted standardized minus crude rates expressed as a percentage of crude rates) is positive from 1914 to 1935, ranging approximately from 20 percent in 1914 down to 5 percent in 1935, to almost nil in 1940, and minus 3 percent in 1945.

Adjusted standardized lung-cancer mortality rates from 1914 to 1950 are plotted on semilogarithmic coordinates in text-figure 2. The points so plotted indicate almost a straight line. The lung-cancer mortality trend is therefore approximately exponential. The magnitude of the increase in recorded lung-cancer mortality, persisting for more than a third of a century, is unequaled by any other cancer site.

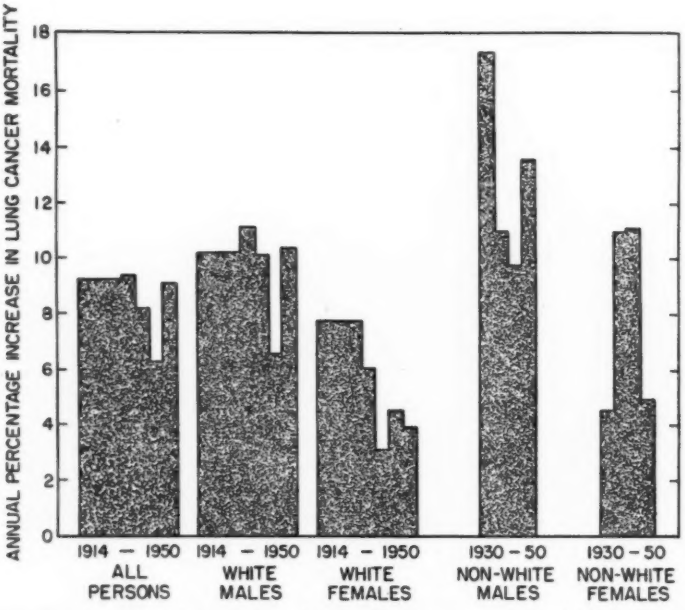


TEXT-FIGURE 2.—Trends of mortality ascribed to cancer of the lung (see table 5 for definition) and cancer of the larynx, total population: Death Registration States, 1914–1930, and continental United States, 1935–1950.

[7] The small negative difference for 1950 may be ignored. It merely reflects the fact that the standard population was the census population enumerated as of April 1, 1950, whereas the 1950 rates are based on the estimated midyear population.

Equivalent annual percentage increases in lung-cancer mortality rates, computed by means of the compound interest equation, are presented in table 7 and text-figure 3. For the entire population of the Death Registration States, the rate of increase was 9.1 percent per annum from 1914 to 1930 and 8.1 percent annually during the next 20 years. The difference between rates of increase during these two time-periods is small but statistically significant ($P<0.01$). In other words, lung-cancer mortality rates are still increasing rapidly, but the rate of increase from 1930 to 1950 was smaller than during the preceding 16 years. The equivalent annual percentage increase declined much more among white females (from 7.7 to 4.4) than among white males (from 10.1 to 9.4). Between 1930 and 1950, mortality rates increased most rapidly among nonwhite males and least rapidly among white females. The rate of increase for white males exceeded that for nonwhite females; it was greater for the total nonwhite than for the total white population. Examination of these data by quinquennial periods shows that rates of increase for white and nonwhite males (and for the total population) declined from 1930 to 1945 but rose again between 1945 and 1950. In contrast, both white and nonwhite female rates of increase decreased during the past 5-year period.

From 1930 to 1950, nonwhites experienced strikingly greater rates of increase in recorded lung-cancer mortality than the white population. The reason for this difference is not readily apparent. One might specu-



TEXT-FIGURE 3.—Annual percentage increase in lung-cancer mortality, by race and sex: Death Registration States, 1914–1930, and continental United States, 1935–1950.

TABLE 7.—*Equivalent annual percentage increase* in recorded lung-cancer mortality by race and sex: Death Registration States, 1914 to 1930, and continental United States, 1935 to 1950*

Years	Annual percentage increase* in lung-cancer mortality				
	All persons	White males	White females	Nonwhite males	Nonwhite females
1914–1930	9. 1	10. 1	7. 7	(†)	(†)
1930–1950	8. 1	9. 4	4. 4	12. 8	7. 8
1930–1935	9. 3	11. 0	6. 0	17. 3	4. 5
1935–1940	8. 1	10. 0	3. 1	10. 9	10. 9
1940–1945	6. 2	6. 5	4. 5	9. 7	11. 0
1945–1950	8. 9	10. 2	3. 8	13. 4	4. 8

*Based on adjusted standardized mortality rates for first and last years of each time interval. The equivalent annual percentage rate increase is 100 r as computed from the compound interest equation

$$R_n = R_o(1+r)^n$$

in which R_o and R_n are mortality rates for the first and last years of each time interval.

†Percentage not computed if R_o or R_n is based on less than 35 deaths.

late that the difference is due to accuracy of medical diagnosis, urbanization, and (if cigarettes are important in etiology) the fact that cigarette smoking increased more rapidly among nonwhites during these years. Information presently available does not permit an adequate evaluation of these or other possible factors.

Mortality rates for cancer of the larynx from 1914 to 1950 are also shown in table 6 and text-figure 2. During this period of 36 years the adjusted standardized mortality rates for the entire population increased by about 80 percent. The rate of increase was greater from 1914 to 1930 than from 1930 to 1950 (table 8). During the latter period larynx-cancer mortality among white females decreased. In 1950 the larynx-cancer mortality rate for white females was 1 percent less than in 1914; for white males it was 96 percent greater than the corresponding 1914 rate. The number of deaths attributed to larynx cancer among nonwhite persons is too small to permit detailed analysis of trends. During the past decade, rates for nonwhite females have been similar to those for white females and rates for nonwhite males have increased more rapidly than the rates for white males. However, in 1950 the larynx-cancer mortality rate

TABLE 8.—*Equivalent annual percentage increase* in recorded larynx-cancer mortality for all persons, white males, and white females: Death Registration States, 1914 to 1930, and continental United States, 1935 to 1950*

Years	Annual percentage increase* in larynx-cancer mortality		
	All persons†	White males	White females
1914–1930	2. 4	3. 0	1. 2
1930–1950	1. 0	1. 0	−1. 0

*See first footnote to table 7.

†Includes nonwhite population. Number of larynx-cancer deaths in the nonwhite population was too small to warrant separate analysis.

among white males was still almost twice as high as among nonwhite males.

Because of the present distressingly high case-fatality rates for cancer of the lung, mortality rates for this disease are thought to provide a reasonably adequate index of liability or risk to disease. This is not true of cancer of the larynx, which is not only more readily diagnosed but more responsive to treatment. Thus, while it appears reasonable to estimate, on the basis of mortality rates, that risk of acquiring lung cancer has increased about 19 times between 1914 and 1950, it does not necessarily follow that larynx-cancer mortality rates are a good index of larynx-cancer incidence rates. This is confirmed by results of cancer-morbidity surveys of 10 urban areas in the United States in 1937 and 1947 (*12*). It was found that the proportional increase in incidence of cancer of the larynx was 1.8 times as large as the proportional increase in mortality. In contrast, for cancer of the lung the proportional increase in incidence was similar to (0.9 times) the proportional increase in mortality.

Summary

Statistics based on medical information recorded on death certificates and representing the mortality experience of the entire United States first became available in 1933. Prior to that date mortality statistics compiled and published by the U. S. Bureau of the Census reflect the mortality experience of the expanding Death Registration Area, which in 1914 comprised less than two thirds of the population of the United States. States admitted to the Death Registration Area early in its development constituted a highly biased sample of the United States. Their recorded mortality rates may be considered as the best available index to national mortality experience but must be interpreted with due regard to their limitations.

In general, the United States has used decennial revisions of the *Manual of the International List of Causes of Death* as a basis for death classification. As a result of periodic revisions of this list and other changes in procedures for coding causes of death, exact comparability of mortality statistics is sometimes lost. This occurred in cancer of the respiratory system.

The earliest mortality data in which deaths attributed to cancer of any portions of the respiratory system can be separately identified are for the year 1914. However, prior to 1930 deaths attributed to cancer of the bronchi, the trachea, or the nasal passages are not identifiable, nor are they included under the heading of cancer of the respiratory system. This is a point that has usually been overlooked in discussions of lung-cancer mortality trends. Fortunately, this point is not of great importance because prior to the decade 1939 to 1948 physicians were much more likely to choose the term *cancer of the lung* for what was, with increasing frequency, later called *bronchogenic carcinoma*.

Recorded mortality rates for cancer of the lung and for cancer of the larynx from 1914 to 1950 were standardized for age and further adjusted

by means of comparability ratios in an effort to overcome some of the limitations discussed above. These comparability ratios take into account only changes in statistical cause-of-death classification procedures—not factors relevant to validity of death certificates, such as improvement in methods of diagnosis, nor the effect of the addition of new Death Registration States. For the years 1914 to 1948, comparability ratios applicable to lung-cancer mortality statistics—to achieve comparability with data for 1949 and later years—fluctuate between 1.000 and 0.926. In view of the many sources of incomparability of International List rubrics, it seems surprising that the correction factors it was possible to compute are so close to unity.

The lung-cancer mortality rate for all persons in continental United States in 1950 was 19 times as great as the corresponding rate for Death Registration States in 1914. These states contained only 62 percent of the population of continental United States in 1914 and in recent years have had significantly higher lung-cancer mortality rates than other states. The estimate of a 19-fold increase in lung-cancer mortality in continental United States from 1914 to 1950 is therefore conservative. The equivalent annual rate of increase was 9.1 percent from 1914 to 1930 and 8.1 percent from 1930 to 1950. The slight decline in rate of increase during the latter period is statistically significant ($P<0.01$). Between 1945 and 1950, the rate of increase for white males and nonwhite males rose again. In contrast, both white and nonwhite female rates of increase decreased. From 1930 to 1950, nonwhites experienced strikingly greater rates of increase in lung-cancer mortality than the white population. The magnitude of the increase in recorded lung-cancer mortality, persisting in an almost exponential pattern for more than a third of a century, is unequaled by any other cancer site.

For cancer of the larynx there was less than a twofold increase in recorded mortality for the entire population between 1914 and 1950. Among white females, larynx-cancer mortality declined between 1930 and 1950 to a point slightly lower than the 1914 rate.

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Biological Studies on the Rous Sarcoma Virus
I. General Introduction
II. Review of Sources of Experimental Variation and of Methods for Their Control[1]

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I. General Introduction

The Rous sarcoma virus (agent of chicken tumor I) (*1*) is of potential value as a research tool for investigating the nature of cancer as well as the mechanisms of carcinogenesis and cell growth. Assuming the eventual availability of highly purified preparations of this virus, its range of potential usefulness extends from the level of intracellular reactions involving macromolecular particulates to that of gross pathologic and clinical manifestations associated with the varied outcomes of interactions between virus and host organism (the chicken). Two of the most important advantages of work with this particular agent are, respectively, its rapidity of action, and the ease with which early neoplastic reactions to measured doses of it can be recognized at localized sites of inoculation in the subcutaneous tissues (*2*, *3*), particularly in the translucent wing web (*4*).

The strongest doses of this virus produce grossly detectable tumors within 5 or 6 days (*2*) and microscopic evidence of the presence of tumor cells can be detected as early as the 3rd to 4th day (*5*) following inoculation of partially purified virus into the subcutaneous tissues of young (2 to 6 weeks old) susceptible chicks. The time required for the induction of tumors in similar animals with the weakest, or minimal, effective doses is, on an average, only 12 to 14 days, although exceptional, resistant individuals may require as long as 30 to 35 days. The maximum period of observation in quantitative biological experimentation with this agent is therefore about 4 to 5 weeks, or only about $\frac{1}{10}$ to $\frac{1}{12}$ the time required for similar experimentation with the most rapidly acting nonviral types of carcinogenic agents [*e.g.*, methylcholanthrene—see reference (*6*)].

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[1] Received for publication March 25, 1955.

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The practical implications of the tenfold greater turnover of available animal housing space, and of the shorter (1/10) time interval between successive steps in a progressive systematic investigation on carcinogenesis, are obvious. In addition, the rapidity of action suggests that the Rous sarcoma virus [as well as other rapidly acting tumor viruses, such as those of the Shope papilloma (*7*, *8*) and some forms of fowl leukosis (*9*, *10*)] may enter directly into the intracellular biological mechanisms concerned with neoplastic transformation. These viruses may therefore be capable of guiding the investigator directly to the basic elements or reactions which are involved in the transition from the normal to the neoplastic state.

Although the Rous sarcoma virus has been known and has been available in the laboratory for over 40 years, experimental work with it has been sporadic and slow to develop on a quantitative basis. The reasons for this are readily apparent from the writings of the earliest investigators who studied this agent, as well as of those who have been concerned with the development of techniques and conditions suitable for quantitative biological experimentation with it (see II for references to literature). In brief, they reside in the extreme variations in biological results which have been found to be associated both with variations among different lots of virus extracted from different batches of sarcoma tissue, and with wide, and sometimes heterogeneous, variations among individual test chickens of a common lot, in response to the same preparation of virus. Operating simultaneously, in the absence of adequate methods for bringing them completely under experimental or statistical control, the several variable factors associated with each of these general sources have made experimental work with the Rous sarcoma virus precarious, and, in the past, extremely difficult if not impossible to repeat, at will, on a strictly quantitative basis. In a discussion of the first general source of variation, written in 1931, Gye and Purdy (*11*) (p. 126) stated that "The variable activity of filtrates is a serious inconvenience in all experimental investigations of filterable tumors; the inconvenience is multiplied ten fold by the instability of the causative agent. . . . . Much loss of time and energy, and a considerable wastage of animals and material occurs in consequence of the unstable, often fleeting, character of the material upon which the induction of new tumors depends." Similar conclusions have also been stated by others (*12*).

In spite of some improvements in methods for the experimental or statistical control of the variations associated with test-animal responses (see II), the problems of laboratory experimentation with the Rous sarcoma virus remained very much the same as stated by Gye and Purdy (see above) until recent years, when stronger virus preparations became available and adequate methods were found for stabilizing and preserving them over relatively long periods of time (see part II).

A review of the known sources of experimental variation and of methods for their control is given in the second (II) communication of this series.

II. Review of Sources of Experimental Variation and of Methods for Their Control

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A. Introduction

Much of the subject matter reiterated herein, particularly that included in the initial section, has been previously reviewed and discussed in detail by a number of writers. The present purpose is therefore not that of presenting an additional primary review of the literature, but of providing a résumé of the information available at the present time with regard to the technical problems and the difficulties encountered in quantitative biological experimentation with the Rous sarcoma virus (*1*), and of methods which have been developed for their control. The references cited in the first section, which deals with variations associated with virus preparations, are primarily to previous reviews or discussions. Original articles are frequently cited, however, by the first investigators to deal with a problem, in which additional discussions pertinent to the present purposes are contained. The subject matter of the second section, concerning test-animal variations and bioassay methods, has not been previously reviewed in detail. The references cited therein are primarily to original reports.

The earliest investigations on this agent, by Rous and his co-workers, were reviewed by Gye and Purdy (*11*) in their book *The Cause of Cancer*, published in 1931. Subsequent reviews of the earlier literature on fowl tumors, including the Rous sarcoma and the agent causing it, were published by Claude and Murphy (*13*) in 1933 and by Foulds (*14*) in 1934. Summarizing discussions have also been presented by Gye (*15*), 1936; Rous (*16*), 1936; and Engelbreth-Holm (*17*), 1938.

The most extensive review of recent years is that published by Harris (*18*) in 1953. Additional reviews or discussions of various phases of the problem have also been presented by the following authors: Rous (*19*), 1943; Duran-Reynals and Shrigley (*20*), 1945; Duran-Reynals (*21*), 1950 and (*22*), 1953; Shrigley (*23*), 1951; Kenzy (*24*), 1952; Carr (*25*), 1953; Oberling and Guérin (*26*), 1954; and Beard, Sharp, and Eckert (*10*), 1955.

B. Variations Associated with Virus Preparations

1. Variations in the Initial Potencies of Virus Preparations

Extreme variations in the initial potencies of filtrates prepared from extracts of different experimentally induced tumors were described in the earliest literature on the agent of chicken tumor I (now generally referred to as the "Rous sarcoma virus") by Rous and his co-workers (*11*, *27*) as well as by subsequent investigators (*11*, *12*, *28*). In general, the more malignant and the more easily transplantable the tumor was found to be, the easier it was to demonstrate a filterable etiological agent. Even with tumors that were readily transplantable, however, filtrates prepared from malignant material "under the best of conditions" not infrequently proved entirely innocuous (*27*). There were even prolonged periods during which no virus at all could be recovered from tumors of lines maintained by serial transplantation in chickens, although, eventually, the property of filterability would reappear (*11*, *14*, *22*, *26*, *28*). Not only could the virus not be recovered from some tumors, but extracts of them sometimes yielded an inhibitory substance which would inactivate added active virus (*12*, *13*).

A technical measure of importance during the early years of work with this virus was the addition to filtrates, before their inoculation into test chickens, of kieselguhr or some other substance capable of producing a nonspecific tissue irritation (*11*, *29*). By this procedure Rous and his associates were able to increase greatly the percentage of experimental fowls which developed tumors, and the resulting growths appeared sooner and enlarged more rapidly. Later, however, after the virus had been propagated in the laboratory for about 18 years, Gye and Purdy (*11*) found that the addition of kieselguhr was no longer essential and that equally good results were obtained with the stronger filtrates, whether or not tissue-irritating substances were added before their inoculation. A corollary to this observation was the fact that the tumors observed at this time were, in general, more malignant and more rapidly growing, and that they produced more potent filtrates on extraction than those

occurring during the earlier years of the laboratory propagation of this virus. Wide individual variations still occurred, however, and some slowly growing, less malignant tumors were encountered which yielded no virus on extraction and filtration (*11*).

Both Rous and Gye and their associates were able, through the selection of the most malignant and the most rapidly growing tumors as sources of virus, to cut down the number of experimental failures (see I) and to make considerable progress in studies on the nature of the agent as well as on host reactions to it. Gye and Purdy (*11*) studied the technical problems further, and described (and classified) various types of experimental tumors produced with the Rous sarcoma virus ranging from clinically and morphologically benign to highly malignant. Although there was found a definite correlation between the type of the tumor and the potency of the filtrate prepared from it, the correlation was not absolute, and "strangely unaccountable exceptions" sometimes occurred. Carr (*12*) studied the relations between tumor structure, age of the tumor (*i.e.*, the length of time it had been growing in its host), and the amount of virus extractable per gram of tumor. From observations on 61 tumors he concluded that the amount of virus extractable was not related to structure *per se*, but showed a very high inverse correlation with the age of the tumor. In general, the older the tumor, the less virus could be recovered from it, and tumors which had grown in their hosts for more than 40 days failed to yield any virus at all on extraction. "Appearance" seemed to be of value "only in indicating that a large tumor of the slow-growing type must be an old one, and hence only a small yield of extractable agent is to be anticipated."

In a recent discussion of the nonfilterable phase of Rous sarcoma, Oberling and Guérin (*26*) pointed out that the variations in the activities of filtrates are related to the conditions of the host with regard to its suitability for the "spread of the virus," and offered the suggestion that the virus may, in different situations, exist in one of two different states, namely, free or masked. Similar changes in the state of the virus had also been suggested by Duran-Reynals and Freire (*30*). A third state in which the virus might be fixed (*e.g.*, adsorbed to red blood cells, or other substances) and nonfilterable, but still capable of inducing tumors, was suggested also by Oberling and Guérin (*26*).

Other factors which have been shown to have an important influence upon the susceptibilities of chickens to the Rous sarcoma virus, and, in instances in which they have been investigated, also upon the amount of virus recoverable from tumor tissue, are: the genetic constitution of the host chicken (*12*, *22*, *24*, *25*, *31–33*); the age of the host chicken (*21*, *22*, *25*); and fluctuations in the level of susceptibility of a given chicken population from time to time (*4*, *26*, *34*). The latter factor has been discussed in relation to possible seasonal effects (*e.g.*, of vitamins and hormones) by Oberling and Guérin (*26*) and Carr (*33*); and in relation to the age of the host, possibly in association with "natural antibodies" in the blood of normal fowls, by Duran-Reynals (*21*, *22*).

Although certain breeds or strains of chickens have been found to be extremely resistant to the virus (*24*, *31–33*), it is usually not difficult to locate highly susceptible strains for propagation and study of the agent. In general, the younger the test host, and the earlier the age of the tumor, the more virus can be extracted from the tumor. Extremely young hosts, *e.g.*, newly hatched chicks, however, may respond to the virus with a hemorrhagic type of lesion rather than by a definitely recognizable neoplastic reaction (*18*, *21*, *26*, *35*, *36*). Fluctuations in population characteristics of test animals, although they do occur, have not been found in this laboratory to be of determining significance with respect to the obtaining of highly active virus suspensions when the chickens are of high average susceptibility and when rapidly growing, highly malignant tumors are used as sources of the virus. Variations in the potencies of different extracts prepared under these conditions may occur over a range of 1 or 2 $\log_{10}$ intervals (*30*, *37*), but the weakest of such materials are still highly active biologically, and are capable of inducing maximal test-animal responses even after being diluted 100-fold or more.

An additional factor of importance in determining the potencies of extracts is the recent finding in this laboratory of a highly significant correlation, below a certain initiating-dose level, between the dose of virus used to initiate a tumor and the amount which can be extracted from it. A full description of these findings is given in an accompanying report (*37*).

2. Variations in Stability of Virus Preparations (Rate of Loss of Biological Activity)

Additional investigations by Gye and Purdy (*11*) revealed another source of variation of even greater technical importance than that associated with tumor type (which could be controlled to some extent through the selection of materials), namely, extreme differences in the stability of the active agent in different filtrates, even among those of relatively high initial potency. As a consequence, different filtrates varied considerably with respect to the conditions under which they could be handled in the laboratory without loss of their tumor-producing properties. The rate at which filtrates lost potency was observed to have "a very large temperature coefficient," and it was this finding which led to the suggestion (*11*), still in use today, of keeping the virus preparations cold (*e.g.*, in a refrigerator or in an ice water bath) whenever practical during their preparation and use in laboratory investigations.

Although most of the filtrates studied by Gye and Purdy (*11*) lost most of their activity within 3 or 4 hours (some more rapidly than others) at a temperature of 37° C., a few were observed which retained their ability to produce tumors for 24 hours, and two were found to remain active after 2 days in the incubator. Using this higher temperature to augment the reactions, further investigations were carried out in a search for substances or conditions which would stabilize the virus by slowing down the rate of loss in activity.

Hydrogen-ion concentration was found definitely to influence the rate of the reaction (*11*) (see also discussion of phosphate buffers, below, p. 292), but the results were variable and an exact correlation of the rate with *p*H could not be obtained. "It is sufficient to repeat the general conclusion of such work, that *incubation* of a somewhat acid filtrate is more rapidly destructive than incubation of the same filtrate maintained neutral, that is, at *p*H 7.4; moderate alkalinity tends to preserve. Extremes in either direction destroy at once."

Measures which reduced "oxygen potential" or inhibited oxidative enzyme action were also observed (*11*) to slow down the rate of loss of activity in incubated filtrates. Hydrocyanic acid (HCN), in particular, was found to be effective in this respect, in a concentration of 1 in 20,000 (*11*). Other substances (*11*, *18*, *24*, *38*, *39*), such as glutathione, cysteine, and sodium hydrosulphite (*38*), have also been shown to be effective in preserving activity of the virus under anaerobic conditions, but Pirie and Holmes (*38*) found the last two materials to be "rapid destructors of its activity" in the presence of air. Since anaerobic conditions are impractical for many types of investigations, *e.g.*, those involving differential centrifugation or fractionation by other procedures, the latter materials, though of considerable interest in studies of the nature of the agent, are not very satisfactory for general use in stabilizing it.

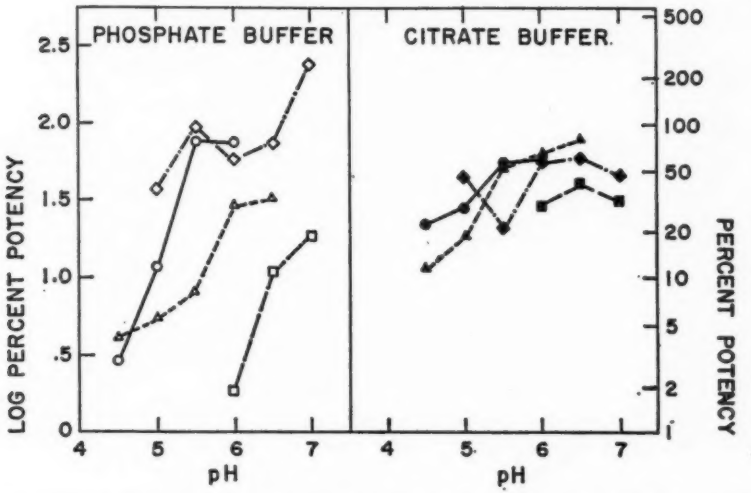
Many investigators have used hydrocyanic acid as recommended by Gye and Purdy, and several have reported results confirming its effectiveness (*18*, *24*, *38*).

While it is quite clear that the persistence of tumor-producing activity during incubation at 37° C. is greater in the presence than in the absence of HCN, at least in the instance of some crude extracts and filtrates, no strictly quantitative studies have been carried out thus far to determine the amount of protection actually afforded by this substance. Exploratory experiments were carried out in this laboratory to test the effectiveness of HCN as a protective agent. "Partially purified" preparations of the virus (microsome fractions separated by differential centrifugation) were used, in contrast to the crude materials which had been previously studied. Although slight protective action was observed in some instances, it was quantitatively unimportant as compared with the total reductions in potency which occurred with some extracts, whether or not HCN was present; *e.g.*, in experiments in which the control preparations lost most of their activity within 24 hours at 37° C. the treated preparations, although slightly stronger than the controls, were nevertheless relatively weak in comparison with their initial potencies. The results of these exploratory tests were not considered as conclusive, but they discouraged further consideration and trial of HCN in this laboratory in the search that was being conducted for an agent of known and simple chemical composition which would provide unfailing protection against loss in activity of partially purified virus preparations.

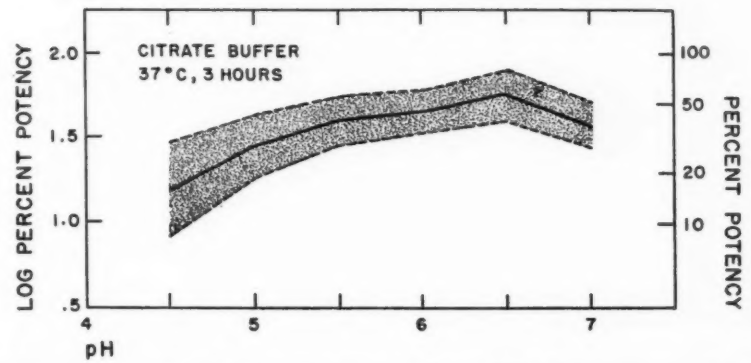
The simplest method found by Gye and Purdy (*11*) to give substantial protection of the virus in filtrates incubated at 37° C. was " . . . to add

horse serum or rabbit serum to the extent of 10 percent." Other sera have also been found to be effective in this respect (*24*). The nature of the protective action of serum was not determined, but it was thought (*11*) to be capable, in some way, of slowing down the action of "ferments" present in the tumor-tissue extracts, which, presumably, might be directly responsible for the degradation of the active principle. The stabilizing action of serum was confirmed by other investigators (*24*), and serum came to be widely used, particularly in diluting preparations of the virus for biological tests. For the latter purpose, concentrations as low as 2 percent have been found to be entirely adequate (*24, 40, 41*). In other experiments, however, such as fractionation of virus containing extracts or of studies on the effects of *p*H or other chemical or physical environmental conditions on the virus, the addition of serum or other complex organic substances as a protecting agent is objectionable, except as a final measure for stabilizing the test materials in preparation for their bioassay. [See Gottschalk (*42*) for studies on the protecting action of serum on another chicken tumor virus.]

Many attempts were made in this laboratory in former years to find a simpler protecting agent of known chemical composition, but without success in the experiments designed specifically for this purpose. Fortunately, however, a highly effective protecting agent of relatively simple and known chemical composition was discovered, inadvertently, during the course of other investigations, namely, those on the comparative stability of the virus in citrate and phosphate buffers (*43*). The original data (*43*) have been replotted, in separate charts, for citrate and phosphate buffers in text-figure 1 in order to illustrate the evidence for a protecting action by citrate, as well as the variability of results obtained with phosphate buffers. In the right chart are shown the relative potencies of 4 different preparations of partially purified virus in citrate buffers at several different levels of *p*H, after incubation for 3 hours at 37° C. The results of the different experiments at a given *p*H varied among themselves no more than was to be expected from the error of the bioassay method, and the data of all 4 experiments are uniformly distributed about a mean trend which may be taken as an estimate of the *p*H stability curve for this agent in citrate buffers—see text-figure 2. In none of the experiments (text-fig. 1) was there an excessive loss of potency, within the *p*H range investigated, during incubation in citrate buffers for 3 hours at 37° C. In contrast, the highly variable results obtained with aliquots of exactly the same virus preparations (originally made up in distilled water and diluted with the appropriate buffers) in phosphate buffers are shown in the left chart of text-figure 1. Not only did one of the preparations (experiment 3) lose most (98%) of its activity during incubation in phosphate buffer at *p*H 6, but also the *apparent* *p*H stability curves obtained in the different experiments varied markedly, and significantly, among themselves. This chart represents an excellent example of the disconcerting variations frequently encountered, heretofore, among different batches of Rous sarcoma virus, and of the consequent lack of



TEXT-FIGURE 1.—Showing the relative stability at different levels of *p*H of partially purified Rous sarcoma virus in phosphate (*left*) and citrate (*right*) buffers (see text). Circles, experiment 1; triangles, experiment 2; squares, experiment 3; diamonds experiment 4.



TEXT-FIGURE 2.—*p*H stability curve for partially purified Rous sarcoma virus in citrate buffers (after standing for 3 hours at 37° C.). The shaded band shows the standard errors of the mean log potencies at successive *p*H levels as determined from the variations between 4 experiments (see text-fig. 1).

reproducibility of results of quantitative biological experiments obtained with this agent at different times under supposedly constant laboratory conditions (see also, discussion of the findings of Gye and Purdy, p. 291). Although the factors responsible for the loss of potency in some materials in phosphate buffers were obviously influenced by *p*H, as had been noted by Gye and Purdy, another factor, namely, the variation in stability associated with the batch of virus, was far more important in determining the rate of loss in activity. The results with phosphate buffers were

therefore spurious as indicators of the effects on stability of hydrogen-ion concentration *per se*.

The systematically high potencies obtained with phosphate buffers in experiment 4 (text-fig. 1) suggest that the virus preparation used in this experiment might have retained its activity for a much longer period at 37° C., and that it may therefore have belonged in the category of the more stable preparations described by Gye and Purdy. There is also the suggestion that some preparations, of which that of experiment 4 is an example, may even show an apparent increase in potency during incubation for short periods in phosphate buffers. Considering the results at all levels of *p*H, the potencies of the suspensions of experiment 4 were slightly higher on an average in phosphate than in citrate buffers. The difference between buffers was on the borderline of significance, but it was not actually significant statistically (*i.e.*, P was approximately 0.05). The results imply the possibility that the dispersion of the virus particles may be increased, or that their combination with an inhibitor may be reversed to some extent in some preparations (*e.g.*, experiment 4), whereas in others (*e.g.*, experiment 3) the virus may undergo some degradation, or masking, during incubation in phosphate buffers. No such indications were apparent, however, in the results for the same virus preparations incubated in citrate buffers.

Similar variations in the stability of different batches of virus have been observed with materials suspended in distilled water and kept for various periods of time, up to 24 hours, at ordinary refrigerator temperature (6°–8° C.) [see footnote 10 of reference (*44*)].

The ability of citrate reagents to stabilize partially purified preparations of the Rous sarcoma virus was substantiated in further investigations (*4*, *44–47*). Methods were also devised for increasing the purity and relative potency of virus preparations (*4*, *46*, *47*) as well as for preserving them at low temperatures (−50° to −76° C.) over relatively long periods of time [up to 2 years (*4*)]. For the latter purpose, advantage was taken of the stability of the agent for at least 3 hours in practically saturated solutions of citrate salts at low temperature (*45*), and the partially purified virus materials were simply suspended in citrate solutions for freezing and storage at subzero temperatures. This eliminates the damaging chemical and desiccating effects met with when other inorganic salts are present and are concentrated in solution during the freezing and thawing processes (*4*). Separate frozen samples of a common lot of virus were further found to have the same potency within the limits of error of the bioassay methods, and to undergo no detectable loss in potency during storage in a CO_2 ice chest for periods up to 2 years (*4*). These findings made possible the use of standard lots of frozen virus, not only for potency reference in quantitative bioassays (see Section C, 4) but also as comparable aliquots of common batches of virus to be studied at different times, under different conditions, or in different laboratories (*4*). The availability of such stable preparations makes it possible, therefore, to eliminate the factor of variation between virus lots, insofar as its influence upon the experimental

results is concerned, by permitting extended or repeated studies to be made on the same preparation of virus.

3. Variations in Degree of Purity of Virus Preparations

A source of variation associated with the virus preparation which remains a major problem in work with the Rous sarcoma virus is that of different degrees of purity of materials prepared from different batches of tumor tissue, either by a constant technique or by different techniques. The solution to this problem is, of course, the final purification of the active agent. The influence of differences between different partially purified batches of virus on experimental results can be controlled by the use of standardized lots, as indicated above, but until methods are devised for obtaining the virus in higher states of purity, the variations between different standard lots cannot be entirely eliminated.

Most of the earlier studies on the Rous sarcoma virus were carried out with crude extracts or filtrates of tumor tissue. Beginning in 1935–38 with the work of McIntosh (*48*), Ledingham and Gye (*49*), Claude (*50–52*), and Amies (*53*) on sedimentation of the virus in the ultracentrifuge, some progress has been made toward its purification either by differential centrifugation alone or by differential centrifugation in combination with other procedures. Most studies during recent years have accordingly been carried out with "partially purified" fractions containing the active principle. [See Harris (*18*) for a recent review of purification methods.] By no method presently available, however, is it possible to obtain practical working quantities of the virus in a high state of purity.

Since successful quantitative biological experimentation with the Rous sarcoma virus requires the use of virus suspensions of relatively high potency, it is desirable to use methods of preparation which can be counted upon consistently to yield such materials. The most satisfactory methods of extraction and preliminary separation of the active fraction that have been found in this laboratory are those which were described in a thesis by Moloney (*46*), and briefly summarized in relation to the descriptions of the preparation of materials CT 559 and CT 581 included in a previous publication (*4*). A more detailed description of these procedures together with additional purification measures, including trypsin treatment as recommended by Amies and Carr (*54*) and by Carr and Harris (*55*), are presented by Moloney (*47*) in a following report dealing with frozen standard virus preparations of a higher degree of purity than those previously employed (*4*).

C. Variations Associated with Biological Responses to the Virus

1. Types of Biological Response

The biological reactions that have been found to be related to strength of Rous sarcoma virus preparation, and which have been employed by various investigators as criteria of relative biological activity are: (*a*) the proportion, or percentage of individuals in a given dose group which show

a positive reaction to the virus, *i.e.*, the development of a tumor (*18, 24, 37, 40, 54–57*); (*b*) the time elapsing between inoculation of the virus and the development of a tumor, *i.e.*, the *latent period* or induction time (*2–4, 10, 27, 37, 57, 58*); (*c*) the rate of growth of the tumor (*11, 27, 59*); (*d*) the size of the tumor at a given time following inoculation, which is a function of the two foregoing reactions (*11, 27, 51, 58, 60–67*); (*e*) the "grade" of the tumor as classified with respect to certain gross clinical and/or morphologic characteristics observed to be roughly correlated with strength of virus preparation (*11, 24*); and (*f*) the number of local ectodermal lesions produced on chorioallantoic membranes of developing chick embryos by dilute suspensions of the virus (*68, 69*). The basic observations underlying the use of all these criteria were originally made by Rous and his associates (*1, 27, 29, 70, 71*). The first, (*a*), represents a response of the quantal type; that is, the proportion of individuals showing an all-or-none qualitative reaction (*10, 72, 73*). Reactions (*b*) through (*e*) are all of the measurable or graded type (*10, 72, 73*). Although variations in the last criteria of this group, (*e*), are only coarsely measurable, nevertheless, the relative frequencies of individuals falling within successive numerically designated categories have been found to provide a fairly sensitive index of relative potency (*24*). The final reaction (*f*) is of the "enumeration response" type, the individual localized lesions being proportional to dose, or number of active physical virus particles in the inoculums (*68, 69*).

Two additional biological reactions of the quantal type, the details of which have not yet been published, have recently come to the attention of the author through personal communications. The first involves the incidence of individuals showing hemorrhagic lesions [*cf.* (*35, 36*)] among older chick embryos injected intravenously with Rous sarcoma virus preparations. A bioassay method based upon this response has been used by Doctors Frederik B. Bang and Wilson H. Y. Lo at the Johns Hopkins University School of Medicine. The second involves the incidence of specific deaths among chicks inoculated intracerebrally, at 1 to 3 days of age, with various preparations and doses of the Rous sarcoma virus. This response has been successfully employed by Doctors Vincent Groupé and Alvin S. Levine at the Institute of Microbiology, Rutgers University.

2. Variations Among Individual Chickens of a Common Lot

Extreme variations among individual chickens in their responses to the same preparation of virus were first pointed out by Rous and his co-workers (*1, 29*). They found some chickens to be completely refractory to their strongest virus preparations, even when diatomaceous earth was added to the filtrate to enhance the biological response to the agent (*29*). Similar findings have been reported by most investigators who have studied this virus, although the frequencies of highly resistant and completely refractory individuals have been found to vary with the age of the chickens at the time of inoculation (*1, 30, 59*), with the experimental strain of chickens (*24, 33*), and with the relative potency, or dilution, of the virus (*57*).

The earliest quantitative studies on the Rous sarcoma virus were carried out with virus suspensions prepared in such a way as to rule out even the most remote possibility of a living cell being present in the experimental material, namely, by filtration of tumor-tissue extracts through bacteria-tight filters (*1*, *11*, 28), or by the use of extracts of desiccated tumor tissue (*28*, *74–76*). Virus preparations of both of these types were considerably weaker in tumor-producing potency than untreated extracts or extracts which had been clarified by passage through coarser filters (*1*, *18*, *28*, *68*) (also, observations in this laboratory). As a result, the strongest concentrations of cell-free virus material employed by the earlier investigators usually failed to produce tumors in 100 percent of the animals within the small test groups used in biological experiments (2 to 8 chickens). In contrast, later investigators, using virus preparations which had been partially purified and concentrated by means of differential centrifugation in an ultracentrifuge (*2*, *4*, *40*, *46*, *51*, *52*, *54*, *55*), observed a lower incidence of completely refractory chickens in results obtained with their strongest virus preparations, and, in some instances, were able to dilute such materials through several serial tenfold steps before the incidence of individuals developing tumors dropped below 100 percent.

Even with the strongest virus preparations available at the present time, however, occasional highly resistant or completely refractory chickens may be encountered in some experimental lots, although not in others (*57*). Information concerning this baffling source of heterogeneity among individual test-animal responses has only recently come to light, through the studies of Eckert, Beard and Beard [(*77*) see also (*10*)] on another chicken-tumor virus, namely, that causing erythromyeloblastic leukosis. These investigators found that in all lines of the different breeds and in the various inbred strains tested by them, the responses of individual chickens were regular (followed a normal curve) and characteristic for a given strain during the early course of an experiment, but, later, some individuals developed an acquired resistance to the inoculated virus which delayed their neoplastic responses to it and introduced considerable heterogeneity into the final results (those made at the end of the observation period). Curves relating the frequencies of positive responses (in probits) with time metameter (log time) were straight lines of constant slope for a given strain during their initial courses, but they became truncated and followed a shallower, more variable slope after a certain period of time. The time of truncation was highly variable, but was significantly correlated with the dose of virus. [For further discussion of this phenomenon see review by Beard, Sharp, and Eckert (*10*).] Similar results have been obtained in this laboratory with some lots of chickens but not with others of the same strain, after inoculation with aliquots of the same standard preparation of Rous sarcoma virus (*57*). The ages of the test chickens and the experimental conditions were the same for the different chicken lots. It is thus apparent that some factor, or factors, which vary from time to time within a given breed and strain, contribute to the presence or absence of heterogeneity among individual chickens in their responses to the Rous sarcoma

virus. When present, the heterogeneous responses increase in frequency as the dosage of virus is decreased (*57*), particularly as it approaches the 50-percent effective dose (ED50). In the proximity of this relative dose the variations among individuals are at their greatest with respect to all of the biological reactions that have been found to be correlated with dose, *i.e.*, latent period, growth rate, relative size at given time, clinical and gross morphologic characteristics, as well as in yield of virus extractable from the resultant tumors. As might be expected, the percentage-incidence response, which is a group reaction, is also highly variable and heterogeneous in chicken lots in which the individuals show heterogeneity, particularly in the response range between 50 and 100 percent (*37*, *57*).

The use of younger (1-day-old) chicks, as recommended by Carr and Harris (*55*) does not reduce variability nor increase susceptibility in the chicken population (New Hampshire Red breed) employed in this laboratory, as it did in that (Brown Leghorn breed) studied by Carr and Harris. Results of two recent quantitative experiments confirmed the finding previously reported from this laboratory (*78*), namely, that very young (4-day-old) chicks were more resistant than older (4-week-old) birds. The recent findings showed them to be more variable, also.

3. Variations Among Different Lots of Chickens of the Same Strain

Variations in the yields of virus from tumors harvested at different times from different chickens of the same strain have already been noted (see p. 288). Although similar fluctuations in the responsiveness of test chickens to the virus have long been suspected, variations in the potencies of virus preparations and the lack, until recently, of stable preparations for use as a standard of reference, have precluded the obtaining of decisive evidence on this likely source of biological variation. However, recent investigations in this laboratory (*4*), resulted in the finding of conditions under which standard preparations of virus of high potency could be preserved over relatively long periods of time (2 years) without detectable loss in biological activity (see p. 294). Concomitant evidence was also presented (*4*) which indicated that different groups of chickens tested at successive times differed significantly in their average levels of susceptibility. The results showed not only highly significant fluctuations about the over-all mean for chicken groups closely adjacent in time, but also significant undulatory changes in the level of average susceptibility over longer periods of times. No evidence of the operation of seasonal factors is apparent in the published data [see text-fig. 1 of reference (*4*)].

Further studies described in a following report (*57*) show that significant differences may occur from lot to lot with respect to heterogeneity and to the extent of the variation among individual chickens within the different experimental lots.

The criterion of biological response employed in the above-cited investigations was a transformation ("metameter") of the latent-period response which has been found (*2–4*) to be suitable for quantitative biological

purposes, namely, the reciprocal of the latent period in days multiplied by 100 to avoid decimal fractional values. Carr and Harris (*55*) "avoided" the use of this and similar graded responses (*e.g.*, the size of tumor at specified time) because " . . . there appears to be no *a priori* reason for assuming that a constant standard could be obtained or that the subsequent comparison would be unaffected by such factors as the season of the year or the nature of the medium in which the virus was suspended." While this statement of factors which may influence the results is unquestionably correct, the implication that the alternative criterion of response which they chose to use, namely, the titration end-point or estimated "minimal infective dose (m.i.d.)," is not influenced by exactly the same factors is entirely unjustified in the light of all evidence available at the present time. The concept that dilution end-points obtained with the percentage response are dependent only upon "a Poisson distribution of virus particles" in the inoculums, that they are not influenced by animal variations or by the nature of the suspending medium, and that they therefore yield absolute estimates of the amount of virus (*55*), has not been substantiated in any investigation that has been reported thus far in which other viruses have been titrated in living animals [see Beard, Sharp, and Eckert (*10*) for a recent review of this subject]. Since Carr and Harris do not give experimental evidence in support of their assumptions, it would seem wiser for the present, pending the appearance of evidence to the contrary, to regard the percentage responses and the end-point-doses inferred from them (such as m.i.d. and ED50, which are, in reality, simply transpositions of variable biological responses to their equivalent positions on a fixed dilution scale) as having the same inherent variability as all the other biological manifestations of virus action, and to follow the same precautions with regard to their control as would be taken for the other responses.

4. Control of Test-Animal Variations in Biological Experiments

The choice of criterion of biological response as well as of method for its control are, of course, governed by the purpose of the experiment and by the biological materials that are appropriate to it. Two general types of investigations may be recognized for the purpose of this discussion, namely: (*a*) Those involving the nature and reactions of the active agent *per se* which require highly accurate comparisons of relative potencies among different virus materials (*e.g.*, bioassays); and (*b*) those concerned with the comparison of host reactions to the virus, either among similar groups of animals subjected to different experimental conditions, or among different types of test animals observed under constant, or comparable technical conditions.

(a) *Potency Comparisons Among Virus Preparations*

1. Experimental designs.—For quantitative potency comparisons it is necessary to eliminate as much of the biological variation as possible through experimental design or by means of appropriate statistical pro-

cedures, and to have some means of estimating the residual (uncontrollable) variation so that the limits of "experimental error" will be known. The earliest studies on the Rous sarcoma virus were carried out at a time before the development of modern bioassay methods, and efforts were not made to determine the magnitudes of the experimental errors. Nor, were estimates made of the slopes of the curves relating dose and response, so that observed differences in biological response could be converted to actual numerical estimates of relative potency. Nevertheless, the measures which were introduced by the earlier investigators for the control of wide and heterogeneous chicken differences, through experimental design, were as efficient as any that could have been devised under the conditions prevailing at the time.

The first principle of control, involving the multiple inoculation of materials into different but comparable sites on the same chicken, was introduced by Rous and his co-workers (*27*, *63*, *70*) during the early course of their studies. It involved the injection of "control" and "test" preparations into comparable sites (in the breast regions) on opposite sides of the body of the same chicken, and the replication of this basic procedure for each chicken within the same test group. Their earliest recordings of results [*e.g.*, (*27*, *70*)] were in the form of charts showing the times of appearance of the tumors (latent periods), the relative sizes of the tumors at successive time intervals (an expression of growth rate), and the relative sizes of the tumors at the end of the observation period (size at standard time). In addition, chickens and sites which failed to develop tumors were indicated on the charts, so that tumor-incidence responses for groups of chickens were also inherent in the published data. Although all of the individual responses varied widely for the same material among different chickens, the comparisons of the response made between materials on the same chicken were consistent and were devoid of the wide fluctuations due to differences between chickens.

Gye and his collaborators (*11*, *58*) applied this same balancing principle in assay procedures involving the size, at specified time, of tumors resulting from inoculation of virus into breast muscles. Since, in control studies involving different doses of the same agent material on the same chicken, they had found tumor size to be roughly proportional to dose, they regarded ratios of tumor sizes within chickens as representing rough estimates of actual virus quantity ratios. However, others who used the same method, or modifications of it, regarded the size differences simply as indices of the order of relative potency rather than as indicators of actual quantitative potency ratio values. Gye and Purdy also stated that tumors resulting from inoculations at other intramuscular sites on the same chicken (*e.g.*, in the wing and thigh areas) were likewise proportional to dose, and were intercomparable, irrespective of body location, but Pirie and Holmes (*38*) found that it was "impossible to compare the size of tumor in one bird with another, or to compare a tumor in the breast with one in the leg." The total incidence of tumors among all members of a test group (percentage response) has also been used as an

indicator of biological activity in assays involving this same principle of multiple inoculations on the same host for the control of the differences between chickens (*40,54*). The percentage response was not generally used by earlier investigators, however, obviously because of the interference with interpretation of results caused by the refractory individuals.

A refinement of the experimental procedures involving the above balancing principle, was introduced by Rous (63), in 1913, and was further developed and applied routinely in bioassays by subsequent investigators (*40, 51, 64–67, 75, 79, 80*). It was based upon the intracutaneous, rather than the intramuscular, injection of virus materials into multiple sites on each test chicken. When pullets or adult hens (*40, 63*) were employed, many (6 or more) inoculums could be readily placed intracutaneously in the feather-free skin areas on the ventral surface of the body along both sides of the sternum [*cf.* (*63, 66*—figure 1)]. The resulting tumors remained discretely localized for sufficiently long periods of time for assay purposes and were more accurately measurable than were intramuscular tumors. Moreover, all the intracutaneous tumors arising in these skin areas were comparable within the same chicken, so that many different virus preparations, or several different dilutions of each, could be compared at the same time in each chicken of a given test group. A further modification of this same basic procedure was that of allowing a specified reference tumor on each chicken (produced by a specified virus inoculum) to reach a certain standard size, and to compare other tumors on the same chicken with it at this particular time (which varied among different chickens) rather than at a standard time for all chickens (*65*).

The subsequent evolution of experimental designs based upon similar balancing procedures appears to have paralleled increases in potency of available virus materials. The relatively weak preparations of the earlier investigators usually gave less than maximal biological responses for reactions of all types, including percentage responses, even when the strongest concentrations were used. Such materials were therefore not diluted before being tested for biological activity, but were inoculated in one concentration only, the strongest available. Subsequent investigators, using stronger materials, found it necessary, however, to dilute their materials to avoid maximal biological reactions.

Using multiple intramuscular sites of inoculation on each chicken, Amies and Carr (*54*) prepared serial tenfold dilutions of both control and test materials and inoculated equal dilutions of each, according to the foregoing balanced design, into a group of chickens at each successive level of dilution. Both dilution-end-point (m.i.d.) and tumor-size information were used in their interpretations of the data. Tumor size was charted diagrammatically in terms of size categories designated by different numbers of plus signs.

Claude and Rothen (*40*) injected successive serial dilutions of their materials into multiple intracutaneous sites on each chicken, and likewise used both dilution-end-point and tumor-size information for interpreting

the results. For some purposes, Claude (*51, 64*) used an "index of tumor-producing activity" derived by combining both percentage-incidence and tumor-size information [*e.g.*, percent incidence × cross sectional area of tumor, the latter being represented by the product of two measured diameters (*51*)].

A method employing similar multiple-site balancing procedures, but involving a different type of response, the latent period (*2, 3*), and a different host tissue, the subcutaneous tissues, was described by the author and collaborators (*81*). Two successive tenfold dilutions of each of two materials, a control ("standard") and a test ("unknown") preparation, were inoculated at different sites on each of a group of chickens according to a Latin-square design. Responses to the two different doses of each material were used for determining the slopes of the dose-response curves. The latter, together with the differences between materials were then employed for estimating the actual numerical potency ratios and their standard errors.

Although they provided a means of fairly efficient control of the heterogeneous chicken variations for the purpose of quantitative bioassay during a developmental period in which better control measures were lacking, procedures based upon multiple site inoculations were limited in practical applicability because of the limited dose span (about 2 $\log_{10}$ intervals) which could be tolerated on a given chicken without disturbance of the balanced design by certain complicating factors. Thus, tumors arising early as a result of stronger inoculums might spread to adjacent areas, or cause death of the host before other, weaker inoculums had had full opportunity to produce their effects. The multiple site designs were also less than ideal, statistically, because of systematic differences among responses at different body locations (*e.g.*, wing, breast, thigh), as well as of the probability of some degree of interaction occurring among responses at different sites in the same host [see Beard, Sharp, and Eckert (*10*) for a discussion of this factor in relation to other viruses]. In addition, Rous sarcoma virus inoculated at one site in extremely young (1-day-old) chicks may localize and produce a tumor at another, remote site which has been injected with salt solution only (*33*). This phenomenon has not been observed, however, in older test chickens, such as those that have been used in multiple-site assays.

Carr and Harris (*55*) described procedures for assay of the Rous sarcoma virus using one-day-old chicks and one inoculation site per chick (the thigh muscle). It is the same in principle as that most commonly employed for the titration of infectious animal viruses, namely, the inoculation of serial tenfold dilutions of virus into successive groups of test animals and determination of the titration end-point. Carr and Harris used 3 to 8 chicks per dilution group and employed, as the titration end-point, the "minimal infective dose (m.i.d.)," which was the highest dilution at which a tumor was produced.

Kenzy (*24*) also used one inoculation site per chicken in titrating Rous sarcoma virus activity. The dilution end-point employed by him was the

greatest dilution capable of producing "at least a 2+ type tumor within 30 days in 50 percent of the inoculated chicks," as interpolated by the method of Reed and Muench (*82*). The end-point dilution obtained in this way was also called a "minimal infective dose (m.i.d.)," although in this case it represented an average minimal infective dose computed from the responses of all chickens within the significant response range (the range of transition from 100 percent to 0 percent), rather than the highest single dilution observed to produce at least one tumor, which was the sense in which Carr and Harris (*55*) used the same term. "Minimal effective dose" has also been used to indicate the dose which is just sufficient to affect all of the test animals in a group. Such varied uses led to the rejection of this term altogether in the field of biological standardization, in order to avoid confusion as to its specific meaning in a particular case [*cf.* (*83*)].

In addition to the titration method employed by Kenzy (*24*) for the estimation of virus quantity, a tumor index based upon the grading of tumors with respect to clinical and morphologic characteristics was also described by him (*24*) for the evaluation of neutralizing antibodies effective against the Rous sarcoma virus.

A bioassay method, employing one inoculation site per chicken but involving the latent period instead of the tumor-incidence response, has been described in a recent report from this laboratory (*4*). Further studies on the same method are presented in a following report (*57*). Because of heterogeneity of susceptibilities of test chickens of the strain (commercial source) employed in this laboratory, the tumor-incidence response has proved less satisfactory for general use in quantitative bioassays. It can be used, however, provided a sufficient number of animals (10 or more) are included within each dilution group and provided a sufficient number of dilution groups are tested with the control (standard) virus preparation so that heterogeneity may be detected, and allowed for, when it occurs (*37*, *57*).

A method which promises to be of great value in the analysis of tumor-response data of the graded type, when the latter are complicated by heterogeneity of individual responses, is that for the analysis of truncated normal distributions which has been described by Bliss (*84*) and Ipsen (*85*), and applied to tumor-response data by Eckert, Beard, and Beard (*77*). The application of this method to the analysis of latent-period data obtained with the Rous sarcoma virus, using one inoculation site per chicken, is described in a following report (*57*).

2. Principles of potency estimation.—It is not the purpose here to discuss details of biometric procedures, but merely to reiterate the principles of quantitative measurement that have been applied to the study of various viruses, including that of Rous sarcoma [see Beard, Sharp, and Eckert (*10*) for a recent discussion of this subject].

Although the terms "titration" and "bioassay" have both been used in a general sense to include all procedures by which "potency," or "biological activity," is inferred from biological response, each has also taken

on an additional, more restricted usage in the particular field or fields in which it is preferentially applied. Both terms are used in the following discussion with the particular connotation each has acquired in its specific field of application. The restricted meanings are defined together with the descriptions of the principles to which they are applied.

a. Enumeration of active virus particles by biological methods.—One of the first of the quantitative methods of virus measurement to be developed was the enumeration of active particles of bacteriophage (*86*) along the lines that had been established earlier for the enumeration of bacteria in a suspension (*87, 88*). The principles involved in investigations of bacteriophage have proved to be applicable in studies of other viruses, and procedures employing both 1) plate counts, and 2) dilution methods, have been devised for use with many of the agents.

(1) By the plate-count method for work with bacteriophage, individual foci of activity of individual physical particles are produced by inoculating measured quantities of dilute suspensions of the virus onto layers of bacteria growing on solid culture media (*89*). Under *ideal* conditions the numbers of plaques, or clear areas representing foci of lysed bacteria, indicate the numbers of unit-infectious particles of virus in the measured inoculums. Although under some conditions counts obtained by this method may closely approach those made by direct counting under an electron microscope (*90*), caution is usually exercised in interpreting the counts as representing absolute numbers of unit particles because similar plaques can be produced also by extremely small clumps of virus if the particles are not completely dispersed. Such plaque counts are directly proportional to the amount of virus (*89*), however, and they yield valid estimates of ratios of potency among different preparations of the same type of bacteriophage tested under the same technical conditions. A similar method for the enumeration of animal virus particles by inoculations onto monolayers of cells in tissue culture has recently been described by Dulbecco (*91*). Analogous procedures have also been based upon the counting of local lesions on plant leaves produced by plant viruses (*92, 93*), and of pocks produced on the chorio-allantoic membrane of developing chick embryos by animal viruses (*68, 93, 94*). The latter natural test surfaces show considerable variation among counts on different biological units (*e.g.*, leaves, plants, membranes of different eggs, etc.), which results in deviations from theoretical expectancy (based upon the Poisson law) of both the variance of the counts (*95*) and of the slope of the curve relating the dose (dilution) with average number of lesions (*96–98*). In addition, the "plating efficiency" is usually too low in the instance of such natural test surfaces for results obtained with them to be interpreted in terms of absolute numbers of virus particles. Nevertheless, the counts remain highly correlated with dose, and can be used as an "enumeration response" in much the same way as any other biological response for comparing relative potencies (*cf. 98*), *e.g.*, by the principles described in the following section. Under ideal conditions such as those usually obtainable for bacteriophage assays,

the counts of local plaques have an inherent precision which is greater by about 1 power of 10 than results based upon other types of biological responses (*99*). Even with the complicating effects of biological variations between unit-test surfaces (*e.g.*, leaves, plants), and with the low "plating efficiency," assays of plant viruses based upon the "enumeration response" are still several times more precise than are assays of the same materials made with other types of biological response, *e.g.*, the quantal response [*cf.* (*99*)].

(2) The dilution method of estimating numbers of virus particles involves the chance distribution of particles in dilute liquid suspensions and the chance presence or absence of unit physical particles of virus in small, constant, subsample volumes (*i.e.*, "inoculums") which are subjected to biological tests. Assuming a random distribution of the particles in the initial undiluted suspension, and in all dilutions and subsamples thereof, it is possible under proper conditions to estimate the average number of active particles per inoculum from the proportion of biological test units which show a positive (or negative) reaction to the unit inoculums, on a basis of the Poisson law—see (*93*) for discussion. Where significant variations do not occur among the "susceptiblities" of biological test units, and where the chances are high of each active particle coming into contact with a susceptible host cell (*e.g.*, inoculums of bacteriophage in unit volumes of bacterial suspension), the enumeration of active virus particles by this indirect method may give results approaching absolute counts [*cf.* (*10*)]. However, as is usually the case when the test units are the higher living organisms, such as plants or laboratory animals, the variation among individual "susceptibilities" and the sometimes extremely low probability that a given virus particle will come into contact with a susceptible host cell combine to invalidate this indirect method as a means of estimating *absolute* numbers of virus particles (*10*). Nevertheless, the numbers of "infectious units" of bacteriophage or other viruses determined by this method are quantitatively related to dose (*i.e.*, to actual numbers of active physical units) and the frequencies of positive (or negative) reactions among similarly treated biological test units may be used as a biological response for relative potency determinations made by other principles (see following sections).

The determination of particle number by either the plate count or the dilution procedure is generally referred to as a "titration," and the estimated number of particles per unit volume as the "titer."

b. Determination of relative potencies of virus preparations in terms of biological, or "infectious," units [*i. e., by reference to a "biological standard"* [*cf.* (*72*)]].—By far the most widely used method for titrating animal-virus activity is that employing the inoculation of serial dilutions of virus (usually in tenfold steps, but sometimes in 2-, 3-, or 4-fold steps) into successive groups of biological test units, and the determination, from the frequencies of positive reactions, of that dilution which corresponds to "one infectious unit." Technically, the method is patterned after that used for the enumeration of bacteria by the dilution method discussed

above. Although earlier investigators regarded the "infectious unit" estimated from responses of laboratory animals as identical with physical units of virus [*cf.* (*10*)], most workers at the present time consider such "infectious units" as having relative significance, only, with respect to quantitative interpretation. The wide differences in end-point dilutions obtained in different types of animal hosts with the same virus preparation, or even in the same type of host at different times, or under different experimental conditions, is widely recognized. So too is the wide discrepancy between animal "infectious units" and physical particles, where both have been determined in correlated studies [*cf.* (*10*)]. The confusion regarding the interpretation of the biological results, and the persistence of some investigators in accepting them as indicators of absolute numbers of virus particles, arises from the fact that in certain instances, particularly in the plant virus field and in the instance of some highly infectious animal viruses, the curve relating the percentage incidence response and the dose of virus approximates closely a theoretical Poisson curve for the distribution of one-or-more particles in unit volumes of a suspension. Purely on a basis of the similarity of observed response curves to a well-known mathematical model, therefore, animal responses are considered, by some, to have absolute meaning in terms of numbers of physical particles.

Beard, Sharp, and Eckert (*10*) have recently reviewed this controversial subject and have shown, convincingly, not only that "infectious units" based upon the responses of living animals can have no absolute meaning, but also that the Poisson "one-or-more particle" curve does not even provide a close approximation to the biological results obtained with some animal virus-host systems. The Poisson curve cannot be used as a mathematical model in these instances, therefore, even for the interpretation of potencies in relative terms (*e. g.*, by the principles described in the following section) since its use would require that the slope of the dose-response curve remain constant (within limits of chance variation) at a predetermined value for all groups and lots of test animals, and for all experimental situations. Such is not the case in actual experience with animal viruses titrated in living animals [*cf.* (*10*)].

An alternate mathematical model, namely, the normal curve, has been shown (*8–10, 37, 100, 101*) to be satisfactory for the interpretation of quantal-response (*e.g.*, percentage-incidence) data observed with most animal viruses that have been examined in this respect, as well as in the case of certain plant viruses (*97*). This model has the advantage that its parameter for the slope of the dose-response relationship is not fixed, or constant, but is free to assume any value dictated by the biological observations themselves. Even in instances where the data are actually better fitted by a Poisson curve, a normal curve of standard deviation 0.5 (in log dose units) gives a sufficiently close approximation to the results for most practical bioassay purposes (*10, 99*). It should be emphasized that the particular usage of a mathematical model being considered here pertains to its practical application as "a sufficiently close approximation" for the purpose of judging relative potency relationships and their limits

of chance variations, and that it has no theoretical meaning with regard to the nature of underlying basic biological mechanisms which may contribute to the final, or over-all pattern of the variations among separate animals, or other biological test units. It is a well-known fact that the more factors (sources of variation) which contribute to total variation, regardless of differences in the fundamental distributions of the separate variates, the more the total variation involving the simultaneous variations of all factors tends to approximate a normal distribution curve; and, the greater becomes the variance of the distribution. The addition to other, more basic sources of chance variation of the factor of variation between individuals in their relative susceptibilities to an agent might be expected, therefore, to obscure other component systems of variation, such as the Poisson, which are predictable on theoretical grounds (*102*). The added source of variation would also be expected to increase the total variance above that derived from limited theoretical considerations involving only the chance contact between virus particles and individual cells.

The logic of the application of the relationships of a normal curve to the interpretation of biological data obtained with viruses is based upon these facts. It is unfortunate that its application to practical problems of bioassay has been misinterpreted by some (*102–104*) to represent the proposal of an alternate "theory" regarding basic mechanisms of virus infection, under ideal conditions, and that its use in the analysis of viral data has been judged "untenable" and thereby discouraged.

In actual practice, interpretations of titration data obtained with animal viruses are usually made without reference to a mathematical model and without regard to theory of underlying mechanisms of variation. The information extracted from the experimental data is simply the interpolated dilution which would be expected to yield exactly 50 percent positive responses (*i.e.*, ED50), or its reciprocal which is the "titer" of the virus preparation [*i.e.*, the number of ED50 units contained in a unit (inoculum) volume of the original undiluted sample]. Estimation of the 50-percent point dilution (ED50) is made in most instances by the simple procedure described by Reed and Muench (*82*). This is a method of interpolation which applies to ED50, only, and is not based upon a particular mathematical model. Its use requires only [3] that the responses to dilutions (usually log dilutions in the case of viruses) be symmetrically distributed about ED50.

[3] Although estimates of ED50 are accurately made by the method of Reed and Muench (*82*) when responses to several dilutions are available within the significant response range (between 0 and 100 percent), neither this nor any other method can accurately locate ED50 when a tenfold-dilution series is used and when the slope of the dose-response curve is sufficiently steep so as to yield only one, or no group responses intermediate between 0 and 100 percent. For example, in the latter instance the only estimate by any method is the mid-point between the adjacent dilutions which yielded 0 and 100 percent responses, respectively. Yet, one frequently encounters in virus literature the statement that ED50 values observed under these conditions were derived "by the method of Reed and Muench." Either the results in such instances should be recognized as rough approximations only, or, if greater accuracy is actually desired in the estimation of ED50, smaller dilution increments than tenfold should be used for the titrations if the slope of the dose-response curve is relatively steep. Only under this condition will the application of a statistical method, such as that of Reed and Muench, improve the accuracy of the interpolated 50-percent point dilution. [See references (*83, 105, 106*) for reviews and discussions of other methods of estimating ED50.]

The analysis of results in terms of biological units or titers, *per se*, is used principally for comparisons of infectivity among viruses of different types, or for comparing the susceptibilities of different types of hosts (or tissues) to the same virus. Although its use in the field of virology is broader in its scope of application, it is analogous to the use in the field of pharmacology of the 50-percent lethal dose (LD50) for general comparisons of toxicity among dissimilar materials. For example, the LD50 of an extremely toxic substance may be measured in micrograms, whereas a less toxic material may be reckoned in terms of milligrams, or, a relatively nontoxic substance in grams. Comparisons among ED50 values (or other criteria) and inferences with regard to potency are also made in the field of pharmacology in relation to substances which exert a similar pharmacological effect, although they may differ in actual chemical composition. No other means exists for comparison of the biological effects of such substances. Gaddum (*107*) refers to such comparisons as "comparative assays," in distinction to "analytical assays" which represent determinations of accurate quantitative potency relationships between unknown and standard preparations of the same active compound (see following section). Commenting upon them, Gaddum (*107*) states that "Comparative assays have no satisfactory logical basis . . . and their only justification lies in the fact that the greatest contributions of pharmacology to medicine have been founded upon them. Under the name of screening tests they have led to the synthesis of many potent drugs, but their results have not the same general validity as the results of analytical assays." The argument is much the same for the use of the biological unit (*e.g.*, ED-50) as a basis of reference in fundamental biological investigations on viruses.

c. Determination of relative potencies by reference to standard virus preparations.—Some investigations on viruses require quantitative estimates of potency comparable in accuracy to those required for the purpose of biological standardization. Examples are: fine differentiation among fractions in purification studies; and the correlation of biological properties with physical and chemical properties [*cf.* (*99*)]. The methods that have been applied to the precise bioassay of viruses [when the more accurate procedure of particle enumeration (see above) is not applicable] are adaptations of the methods which have been developed in the field of pharmacology for the biological standardization of drugs, vitamins, hormones, and other compounds [*cf.* (*72, 83, 99, 107–111*)].

The principle involves the adoption of a particular preparation of an agent as a standard of potency reference, and the assay of similar materials of unknown potency in relation to it on a basis of the respective responses of comparable groups of biological test units. The latter are drawn at random from a common lot, the known and unknown materials being tested at exactly the same time under exactly the same technical conditions. In this way, all factors which contribute to variation are controlled experimentally, with the exception of those due to differences among individual biological test units. The latter are controlled by statistical

methods, both in the design of the assay experiment and in the subsequent analysis and interpretation of results. The potency of an unknown is then expressed in terms of percent (or as a ratio) in relation to that of the standard preparation. Although for the purpose of standardizing drugs or other compounds on a national or international basis it is necessary to have a stable preparation for use as "permanent standard," the same analytical bioassay principles can be used for comparing the potencies of any two or more biologically active materials, even when they are not stable, *i.e.*, when they can be maintained in the laboratory for only relatively short periods of time. Thus, a control preparation of a relatively unstable virus can be used as the "standard" (*i.e.*, as the reference material of 100-percent potency) in an isolated experiment involving the assay of one or more test preparations ("unknowns") in relation to it [see (*4, 10, 37, 99*) for additional discussions and references to applications of the principle in the assay of viruses].

It is this principle of analytical assay which, because of its extensive use in the field of biological standardization, has come to be identified with the more restricted usage of the term "bioassay." Application of the principle is the same for all types of biologically active agents, irrespective of the particular nature of the biological response and regardless of whether it is of the quantal (all-or-none) or graded (measurable) type.

When ED50 or titer values have been determined for similar materials in comparable random groups of biological test units, their ratios represent ratios of relative potencies. In the assay of viruses, it is usually the logarithm of ED50 that is estimated from the experimental data. The differences between log ED50 values therefore represent logarithms of potency ratios, and, antilogs of the latter give the potency ratios in arithmetic terms. The direct comparison between ED50 estimates is possible because they represent interpolated doses (or dilutions) which would be expected to produce a constant biological response (positive reactions in 50 percent of the biological test units); *i.e.*, the constant response is determined in relation to dose in each instance from group responses at 2 or more dose (or dilution) levels giving results within the significant response range.

In assays involving responses of the graded type, it is also necessary to have results at 2 or more dose (or dilution) levels within the significant response range (*i.e.*, the range within which dose and response are highly correlated), in order that observed differences in biological response can be converted to their equivalents in terms of dose (or log dose) differences. Usually, there is no certain level of a graded response which has a particular meaning (such as the 50-percent quantal response) and which is chosen as a constant point of reference for comparing the relative doses which produce it. Rather, graded responses are compared by determining the dose-response curves for the respective materials and estimating the relative potency relationships between them by appropriate graphic or algebraic procedures. Similar procedures may also be applied to the

analysis of quantal response data, if so desired. (See references to methods, cited above.)

The accuracy of potency relationships estimated by the "analytical assay" procedure is a function of the total numbers of biological test units included in the individual bioassay experiments. It is limited only by the technical feasibility and the financial practicality of the numbers, which can be employed in routine tests [*cf.* (*99*)].

(b) *Comparison of Host Responses*

Methods for the control of biological variations in the study of host response are similar in principle to those described for assays of the virus in the preceding section. If the reactions among different host groups do not differ very markedly in their qualitative characteristics, then any one or more of the several types of tumor responses already described (p. 295) might be used for the group comparisons. On the other hand, if the lesions differ qualitatively [as for example the hemorrhagic lesions of newly hatched chicks (see p. 290) in comparison with the tumorous growths of older birds] only one type of criterion has been found to be satisfactory for their comparison, namely, the relative dose of virus which is required to produce a characteristic biological response in 50 percent of the inoculated individuals (*i.e.*, ED50). It is understood, of course, that any such comparisons among different types of hosts must involve inoculums of exactly the same virus preparation.

In experiments in which comparable random groups of hosts can be used for each of the conditions under test, *e.g.*, "control" and "treated" groups, and where the responses are similar in nature but differ in degree, experimental designs and analytical procedures identical with those described for bioassays can be employed [*cf.* (*112*, *113*)]. Furthermore, the relative effects of the various experimentally controlled conditions can be expressed in quantitative terms in relation to comparable changes in dose which would be required, on an average, to bring about the same differences in host response. If the experimental conditions themselves do not increase the variability among individual hosts, or produce secondary biological effects (*e.g.*, loss of weight and debilitation) which complicate interpretations of cause and effect relationships of the results, the effects of the conditions should be determinable with a precision approaching that characteristic for bioassays by the same experimental design.

Many biological problems, on the other hand, require comparisons among groups of chickens which are not random samples of the same lot. These range from sex differences within a common lot or minor age differences within the same strain, to extreme age differences or differences between strains and breeds. Under certain conditions (*22*) the Rous sarcoma virus can be transmitted to closely related fowl species. Host comparisons could, therefore, involve differences in species between test groups. Where differences in qualitative characteristics of the response are not involved, the same bioassay designs as above may likewise be used

for comparing host responses [*cf.* (*78*, *113*)]. Like the "comparative assay," however, the results do not have the same "general validity" as those involving comparable random groups of hosts.

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Biological Studies on the Rous Sarcoma Virus. III. The Recovery of Virus From Experimental Tumors in Relation to Initiating Dose[1]

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Extreme variations in tumor-producing activity among extracts prepared from different source lots of tumor tissue have long been a problem in work with the Rous sarcoma virus [*cf.* (*1*)]. Two factors have previously been shown to have an important influence upon the amount of virus that can be extracted from Rous tumors: *a*) the age of the host chicken in which the tumor tissue is grown (*2*), and *b*) the age of the tumor itself; *i.e.*, the length of time it has grown in its host (*3*). In general, the younger the host, the more the virus that can be extracted from the tumor tissue, although extremely young chicks (newly hatched) may develop hemorrhagic rather than typical neoplastic lesions in response to the virus (*4*). In like manner, the younger the tumor, the more the virus that can be extracted from it. Carr (*3*) obtained maximum yields of virus, and found no correlation with tumor age, during the first 13 days of tumor growth. Tumors that had grown for 14 to 22 days, however, yielded slightly less virus on extraction; and those which had grown for 22 to 40 days showed a striking inverse correlation between tumor age and amount of extractable virus per gram of tumor tissue. Tumors that had grown for more than 40 days, even in young susceptible hosts (6 to 9 weeks old at the time of inoculation), yielded no virus at all on extraction.

The appearance after longer latent intervals, and the slower average rate of growth of tumors induced by weaker, as compared with stronger, inoculums of the Rous sarcoma virus have long been recognized (*1*, *5*, *6*). The nature of the quantitative relationship between average latent period and relative dose of virus has been investigated in detail (*7*, *8*), and has been used in quantitative bioassays of the virus (*9*, *10*). Observations made during the course of dose-response studies involving a wide range of virus doses (tenfold dilution series over the entire practical working range) indicated that the frequencies of tumors showing the different types of variation that have been noted for the response to this virus (*e.g.*, morphological characteristics, degree of malignancy, rate of growth, latent period)

[1] Received for publication March 25, 1955.
[2] With the technical assistance of John P. Kvedar.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[*cf.* (*1*)] are all related to the dose of virus. This further suggested that the yields of virus from experimentally induced tumors, which are known to be associated with the average tendencies reflected by the above variables, might be more basically related to the strength of the inducing dose of virus. The present investigation was for the purpose of testing this possibility. The experiments and results are described herein.

Materials and Methods

Standard virus preparation.—The standard virus preparation used for initiating the tumors, as well as for potency reference in bioassays of their extracts, was frozen lot CT 559 which was described in detail in a previous report (*10*). All virus concentrations are expressed in terms of relative dilutions of this standard material, the observed potency of which was taken as 100 percent.

Tumor-tissue extracts ("unknowns").—The method of preparing extracts to be tested for biological activity was constant for all tumors with the one exception described below. One gram of tumor tissue was homogenized in 19 ml. of 0.15 *M* potassium citrate solution containing 1 mg. per 100 ml. of hyaluronidase, by blending for 2 minutes in a Waring blendor. The stainless steel blending vessel was kept cool to touch at all times by the external application of a small piece of CO_2 ice, in the region where the cutter bearing passes through the bottom of the vessel. The homogenate was allowed to stand for 1 hour at room temperature to permit action of the hyaluronidase and was then placed in a refrigerator for an additional hour before being blended a second time in the same manner as above. It was next carried through a series of 3 successive centrifugations for removing intact cells, cellular debris, and the larger intracellular particulates and aggregates, as well as free fat globules, which might buoy up intact tumor cells. The first centrifugation was for a period of 30 minutes at 2,600 $\times$ g in an angle head of an ordinary refrigerated centrifuge. The middle half of the supernatant from this run was carefully separated by means of a transfer pipette and subjected to a second centrifugation for a period of 10 minutes at 5,000 $\times$ g in a Spinco ultracentrifuge. Again only the middle half of the supernatant fraction was recovered, in the same manner as above. This fraction was diluted with an equal volume of citrate buffer (0.05 *M*, *p*H 6.7) containing hyaluronidase (1 mg. per 100 ml.) before being subjected to the third and final centrifugation. The latter was in a Spinco ultracentrifuge at 18,000 $\times$ g for about 2 or 3 minutes (a "run-up-and-down"). Most of the supernatant was recovered after the final run, only a small amount (2 to 3 ml.) being left behind to avoid the extreme bottom and top layers. The recovered fraction was further diluted (2.5 fold) to give a final concentration of the extract of 10^{-2} with respect to the number of grams of tumor tissue originally extracted (*i.e.*, the virus material from 1 gram of tumor tissue would be contained in a total of 100 ml. of the suspension). This was the strongest concentration of cell-free extract subjected to biological tests. It was the same as that found to be optimal for the recovery of virus in filtrates by the earlier

investigators; *i.e.*, stronger tumor suspensions tended to clog filters and were less apt to result in strongly active filtrates (*11*). The procedure of filtration for removal of cells was not used in the present investigation for two reasons: first, large quantities of virus (up to 1 or 2 $\log_{10}$ intervals of concentration) are removed by adsorption on the filters, with consequent reduction in the total potency of the extracts [(*11*, *12*), and observations in this laboratory]; and second, filters are variable in their performances (*11*), and an additional source of considerable error would have been introduced into the quantitative estimates of potency.

With the one exception noted below, all tumors had been stored in a CO_2 ice chest for at least 24 hours before being thawed and prepared for biological testing. In order to conserve samples of standard virus, as well as test chickens, a total of 6 to 13 different extracts were bioassayed on the same day in relation to the same sample and dilution series of standard virus. For this reason it was technically necessary to prepare the extracts on one day and to inoculate them into test chickens on the following day. The standard procedure therefore involved the injection of extracts on the day following their preparation. They were kept in an ordinary refrigerator (6–8° C.) during the intervening interval. Previous preliminary studies had shown that preparations of the virus in citrate reagents retained their activity for periods up to 7 days in an ordinary refrigerator, without detectable loss in virus activity.

The exception to the above preparative procedure was that employed for the tumor of chicken No. 727 (see table 2B, p. 325) which grew slowly and failed to reach the size desired for study (that which would yield at least 1.5 grams of recoverable tissue) before it showed signs of retrogression. With the first evidence that it had decreased slightly in size, the chicken was sacrificed, the tumor was immediately extracted, and the extract was inoculated into test chickens. The amount of tissue recovered was only 0.7 grams. This was ground in a mortar with sand and diluted 1/20 with citrate-hyaluronidase solution. The extract was allowed to stand, as above, but it was not centrifuged before being tested. The sample used for inoculation was simply that withdrawn from the middle of the supernatant fluid layer which formed during standing, without further dilution. Previous experience with retrogressing tumors had shown them to be nontransplantable, and the precautions for removal of intact cells were omitted in this instance.

Technical procedures of bioassays.—The method of bioassay involved the inoculation of each test chicken at one site only [the left wing web (*10*)] and the use of separate groups of chickens for each dose of each material tested. The procedures were the same as those described in detail for material CT 581 in a previous report (*10*).

Each unknown (extract) was prepared in dilutions 10^{-2}, 10^{-4}, and 10^{-6}, and 10 chickens were inoculated with each of these dilutions. The volume of the inoculum (0.2 ml.) was constant for all materials and dilutions thereof. From 6 to 13 different unknowns were assayed on a given day. A single dilution series of standard virus (CT 559) prepared on the same

day was employed as the basis of potency reference for all of the unknown materials. Ten chickens were inoculated with each dilution of the standard tenfold dilution series, which extended from 10^{-2} through 10^{-9}. All chickens included within a given assay experiment on a given day were from a common hatching lot.

The diluent used in preparing all the materials for chicken inoculations was 0.05 *M* citrate buffer, *p*H 6.7, containing 1 mg. per 100 ml. of hyaluronidase (*10*).

Both latent-period and tumor-incidence information were used in interpreting the bioassay results. The former was employed exclusively in the instance of strong extracts which gave a tumor incidence of more than 50 percent with the weakest concentration (highest dilution) tested, namely, 10^{-6}. The metameter of the latent period used for bioassay purposes was the reciprocal ($\times$ 100), which has been previously discussed (*10*). On the other hand, the tumor-incidence response (in terms of probits) was employed exclusively for bioassays of materials which gave an incidence of 50 percent, or less, with the strongest concentration (lowest dilution) tested, *i.e.*, 10^{-2}. Materials of intermediate potency yielded information of both types which could be used in deriving estimates of potency. In such cases the average of the separate estimates was used as the potency value for the particular material. All potency estimates were derived by graphic procedures as illustrated in the following section. The standard errors were not determined for individual log-potency ratios, but, judging from previous results (*10*) and analyses (*13*), they might be expected to be about ± 0.2 to ± 0.4 $\log_{10}$ intervals for the stronger preparations, and about ± 0.4 to ± 0.7 for some of the weakest materials.

The potencies of all materials are expressed in terms of percent, relative to the observed potency of the standard, which was taken to be 100 percent in each assay experiment. Differences due to fluctuations in level of sensitivity of different test lots of chickens were therefore eliminated from the potency estimates derived at different times through reference to the standard material within each test lot.

Test chickens.—The chickens were of the New Hampshire Red breed and were derived from a constant commercial source which maintained its own breeding flock. The chickens of the first experiment (serial No. CT 620), which was for the purpose of producing tumors, were 23 days old at the time of inoculation with virus. Those used in subsequent assay experiments (CT 621 through CT 625) were either 16 or 23 days old, in these different experiments, at the time of inoculation.

Experimental Procedures and Results

Production of Experimental Tumors (experiment serial number CT 620, October 6, 1954).—Because of fluctuations in test-animal sensitivity from time to time (*10*), an effort was made to produce all tumors for subsequent study within a single hatching lot of chickens. The details of the initial experiment for this purpose are shown in table 1. A single dilution series of standard virus (CT 559) was prepared in tenfold steps

as indicated in the first column. The chickens to be inoculated had been previously divided into 2 technical groups, A and B. Equal numbers from each technical group were inoculated subcutaneously, in the left wing web (*10*), with 0.2 ml. of a particular dilution of standard virus. The numbers inoculated at successive dilution levels were varied according to the expected frequencies of tumors based upon previous experimentation with this standard preparation. Only 5 chickens in each technical group, or a total of 10, were inoculated with the 10^{-1} dilution, and none were inoculated with undiluted material (10^{0}) because of the relatively large amounts of standard material required and the necessity of conserving the available supply Experiments carried out a few weeks earlier had shown occasional tumors in the 10^{-9} dilution, and a few tumors were expected out of the 100 chickens inoculated at this dilution in the present experiment. However, the test chickens were more resistant and no tumors were obtained with either the 10^{-9} or the 10^{-8} dilution. Only one tumor resulted among a total of 60 chickens inoculated with the 10^{-7} dilution. This deviation from average expectancy necessitated the supplementing of the tumor material in subsequent experiments as described below.

Neither the differences between tumor responses nor those between the yields of virus from the induced tumors were statistically significant for technical groups, and the data of both have therefore been combined in the last 3 columns of table 1. The technical groups differed in that the chickens of group B had been injected intraperitoneally with a solution of antibiotics[4] on the day before, and for 3 days after, inoculation with the Rous sarcoma virus; whereas those of group A had received inoculations of Rous sarcoma virus only. The tumors assayed for virus content are identified with respect to host and technical group in table 2.

The chickens were examined at daily intervals, and each tumor was harvested as soon as possible after it was judged to be sufficiently large to yield at least 1.5 to 2 grams of tissue for extraction and histological study.[5] The tissues to be used for the biological studies were stored in rubber stoppered bottles in a CO_2 ice chest. With the one exception noted above (p. 319) the time of storage before extraction and bioassay varied from 1 to 55 days (see table 2).

Estimations of potency (Experiment serial numbers CT 621 through CT 625; Oct. 19 through Dec. 8, 1954).—The forms of the dose-response data used for estimations of potency are illustrated in text-figure 1 using the responses to standard virus CT 559 obtained in the preceding experiment (CT 620). The tumor-incidence responses in probits are the same as those tabulated in percent in the last column of table 1. The latent-period data represent the mean reciprocals ($\times$ 100) for the same dose

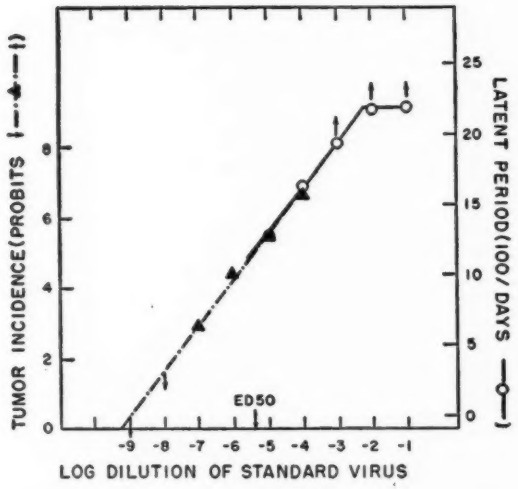
[4] Each chicken of group B received 4 daily doses of 100 units of penicillin G and 0.65 mg. of streptomycin (calcium chloride complex). This division of the test animals into 2 technical groups was exploratory to other contemplated studies and in the present investigation is of incidental interest only, since no significant differences were observed between the technical groups.

[5] The histological studies are being made by Dr. A. W. Hilberg and will be reported upon by him at a later date.

TABLE 1.—*Derivation of experimental tumors tested for virus activity*

Dilution of standard virus (CT 559)	Data of separate technical groups						Combined data		
	A			B			(A+B)		
	Number of chickens inoculated	Number with tumors	Tumor incidence (*percent*)	Number of chickens inoculated	Number with tumors	Tumor incidence (*percent*)	Total number inoculated	Total number with tumors	Total tumor incidence (*percent*)
10^{-1}	5	5	100	5	5	100	10	10	100
10^{-2}	10	10	100	10	10	100	20	20	100
10^{-3}	10	10	100	9*	9	100	19*	19	100
10^{-4}	10	10	100	10	9	90	20	19	95
10^{-5}	15	11	73. 3	15	10	66. 7	30	21	70
10^{-6}	20	6	30. 0	20	5	25. 0	40	11	27. 5
10^{-7}	29*	1	3. 4	30	0	0	59*	1	1. 7
10^{-8}	40	0	0	38†	0	0	78†	0	0
10^{-9}	50	0	0	50	0	0	100	0	0
Total	189	53	.....	187	48	.....	376	101	

*Corrected for one death without tumor during observation period.
†Corrected for two deaths without tumor during observation period.



TEXT-FIGURE 1.—Showing the incidence of tumors (in probits) and the average latent-period response (in units of 100/days) in the initial experiment (CT 620) for the production of tumors. The arrows pointing downward and upward represent 0- and 100-percent responses, respectively. ED50 represents the "50-percent effective dose," which was at dilution $10^{-5.4}$.

groups. These data are chosen for illustration because of the relatively large number of chickens per dose group and the consequent regularity of the biological results. The responses to the standard dose series were similar in the individual bioassay experiments, but the plotted points were subject to more variation about the estimated curves because of the smaller number (10) of chickens per dose group. It is seen that the total range of dilutions available for bioassay purposes covers about 4 to 5 $\log_{10}$ intervals, considering both the latent-period and the tumor-incidence types of response. The former is limited at high doses (low dilutions) by a minimal latent period, which lies between the 4th and 5th days following inoculation (7). It is also limited at the lower doses, in the region of the "effective dose 50" (ED50), since the grouping error of the individual latent periods becomes quite large, and the mean results become unreliable when an appreciable proportion of the individuals in the small experimental group fails to develop tumors (*i.e.*, fails to contribute latent-period values) (7); there is also some evidence in compiled data on hand that the correlation between dose and latent period either breaks down or follows a flatter regression line at these lower dose levels. On the other hand the percentage response is most reliable in the region of ED50 and can be used for estimating potency at the higher dilutions (lower relative concentrations), down to the lowest dose (highest dilution) that produces a tumor in one or more of the inoculated chickens. The tumor-incidence data for small groups of chickens are frequently truncated at the higher percentage (or probit) levels, however, owing to the occurrence of highly

TABLE 2.—*Detailed data of tumors assayed for virus content*

A. Tumors induced in initial experiment (CT 620)

Concentration of standard virus (CT 559)		Expt. Group	Donor chicken		Experimental tumor					Incidence of tumors in dose group	Potency of extract relative to standard virus (CT 559)	
Log Dil.	ED50 units per ml.		Expt. ser. No. (CT)	Leg band No.	Date tested	Time of storage in CO_2 ice chest (*days*)	Latent period (*days*)	Time of growth after gross detection (*days*)	Total time from inoculation of virus to excision of tumor (*days*)	(*percent*)	(Log percent)	Percent
−1	2.5×10^4	A	620	180	10–19–54	5	5	3	8	100	2. 30	200
				50	11–10–54	55	5	4	9		2. 72	520
				107	12–1–54	47	5	3	8		2. 90	790
		B		135	10–19–54	5	5	3	8		3. 00	1000
				133	11–10–54	27	5	2	7		2. 46	290
				39	12–8–54	55	5	2	7		2. 95	890
−2	2.5×10^3	A	620	56	10–19–54	6	5	2	7	100	3. 10	1260
				36	12–1–54	47	5	3	8		3. 17	1480
				72	12–1–54	47	5	3	8		1. 30	20
		B		177	10–19–54	5	5	3	8		2. 56	360
				71	10–19–54	6	5	2	7		3. 38	2400
				43	12–8–54	53	5	4	9		1. 78	60
−3	2.5×10^2	A	620	169	10–19–54	4	6	3	9	100	2. 21	160
				16	11–10–54	25	6	3	9		2. 62	420
				115	12–1–54	46	6	3	9		1. 80	63
		B		51	10–19–54	4	6	3	9		2. 88	760
				127	12–1–54	45	5	4	9		2. 98	960
				143	12–8–54	43	6	3	9		2. 55	350
−4	2.5×10^1	A	620	38	10–19–54	1	7	5	12	95	2. 67	470
				10	10–19–54	1	5	7	12		2. 52	330
				94	12–1–54	43	7	5	12		1. 70	50
		B		41	10–19–54	1	6	6	12		2. 29	190
				13	12–1–54	42	6	7	13		1. 05	11
				98	12–8–54	48	8	6	14		1. 75	56

−5	2.5	A	620	149	10–27–54	6	8	6	14	70	0. 73	5. 4
				183	10–27–54	5	8	7	15		1. 73	54
				91	12–1–54	40	8	7	15		2. 00	100
		B		100	10–27–54	6	6	8	14		2. 28	190
				65	11–10–54	21	8	5	13		1. 57	37
				86	12–8–54	46	10	6	16		. 10	1. 3
−6	2.5×10^{-1}	A	620	150	10–27–54	5	12	3	15	27. 5	−2. 00	0. 01
				132	11–10–54	13	10	11	21		0. 04	1. 1
				158	11–10–54	3	14	13	27		−1. 24	. 057
				171	11–10–54	11	10	13	23		−0. 16	. 69
				165	12–1–54	34	16	5	21		−0. 87	. 14
				60	12–1–54	20	14	21	35		−2. 05	. 0089
		B		108	10–27–54	4	12	4	16		(Negative)	(0)
				116	10–27–54	5	8	7	15		(Negative)	(0)
				19	11–10–54	11	14	9	23		−0. 67	. 21
				184	12–1–54	28	16	11	27		−1. 62	. 024
−7	2.5×10^{-2}	A	620	168	12–1–54	20	10	25	35	1. 7	−1. 43	. 037
					B. Supplementary tumors (see text)							
−5.4§	1.0	R‡	622	538	12–8–54	19	12	10	22	50	1. 00	10
−5.4§	1.0	R‡	622	481	12–8–54	19	12	10	22	50	(Negative)	(0)
−5.7§	6.3×10^{-1}	R‡	621	745	12–8–54	57	12	9	21	33. 3	−2. 85	0. 0014
−5.9†	4.0×10^{-1}	R‡	624	727	12–30–54	0	10	19	29	10	(Negative)	(0)
−6.4†	10^{-1}	R‡	622	480	12–1–54	4	(<27)*	(>3)*	30	10	−1. 62	. 024
−6.9‖	4.0×10^{-2}	R‡	623	174	12–8–54	1	9	18	27	10	−1. 80	. 016

*Tumor, measuring about 1 cm. in diameter, discovered protruding from dorsal surface of wing opposite inoculation site on ventral surface on 27th day. The latent period and the time of growth since first appearance could not be exactly determined.

†Tumor induced with dilution 10^{-7} of standard virus CT 559, but corrected for deviation of ED50 as in §.

‡Reference group of chickens inoculated with standard virus CT 559, which was the standard of potency reference in bioassay experiments CT 621 through CT 624.

§Tumor induced with dilution 10^{-6} of standard virus CT 559, but dilution corrected for deviation of responses of group CT 622 from original group (CT 620), on a basis of the deviation of ED50.

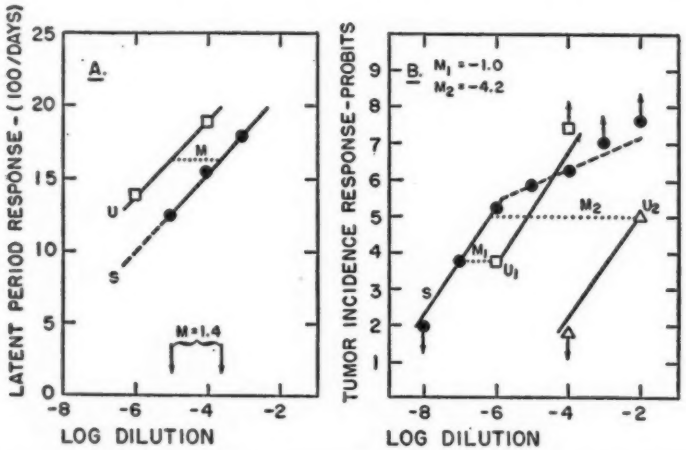
‖Tumor induced with dilution 10^{-6} of standard virus CT 559 in chicken group CT 623, which differed in ED50 from the original group (CT 620) by 1.1 log intervals. Corrected as in §.

refractory individuals in some test lots of chickens. In such instances, the tumor-incidence response is not reliable in the region of the higher incidence values. An example of this is shown in the right chart (B) of text-figure 2.

Since the standard dose-response curve was established on a fairly large number of chickens (80) in each bioassay experiment, the slope of the standard curve could be used for estimating the log-potency ratio of an unknown, even when only one dilution group of the unknown fell within the significant response range (for either the latent-period or tumor-incidence type of response).

The graphic estimation of the log-potency ratio (M) is illustrated for both types of response in text-figure 2. The antilog of the M value ($=1.4$) found in the left chart (A) is 25, and the unknown extract therefore had a relative potency of 25 times that of the standard. In terms of percent it was 2,500 as compared with 100 percent for the standard. The middle curve of chart B shows the tumor-incidence response to dilution 10^{-6} in an assay in which the two lower dilutions (10^{-4} and 10^{-2}) both fell within the significant latent-period range. The value of M determined from the latter data was -1.55, which is within limits of error of the value, -1.0, found from the single tumor-incidence response of text-figure 2B. The response curve furthest to the right in text-figure 2B represents an example in which the strongest concentration (dilution 10^{-2}) of the material gave a response of only 50 percent, and the higher dilutions failed to produce any tumors at all. Only this one point was available therefore for the potency estimation.

Supplementary tumors at low-dose levels.—It has already been pointed out that the same standard preparation of virus (CT 559) was used for bioassay reference as was used in the initial experiment (CT 620) for the

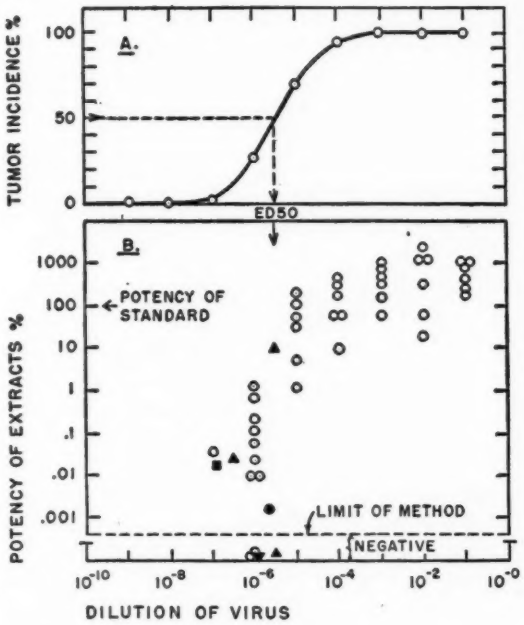


TEXT-FIGURE 2.—Illustrating the bioassay of tumor tissue extracts by graphic methods (see text). S and U represent standard and unknown virus preparations, respectively.

production of tumors. Tumors produced by high dilutions of the standard in the bioassay experiments were therefore saved and added to the initial group of tumors for study with respect to extractable virus content. Fluctuations in the level of chicken-lot sensitivity to the virus made it necessary, however, to make corrections for the actual dilutions of standard which gave rise to the tumors, so that they would conform in level of tumor response to estimated dilutions which would have been expected to produce the same tumor response in the initial lot of chickens (CT 620). This was done by correcting each dilution by the deviation of ED50 for the particular chicken lot from the ED50 value ($10^{-5.4}$) which had been observed for the initial lot (see text-fig. 1). The data for individual tumors in this category are recorded in the lower part, B, of table 2

Interpretations of Results

The interpretations of results of bioassays of the tumor-tissue extracts are more readily presented through reference to the graphic distribution of the potency estimates, in relation to initiating dose of virus, as plotted in text-figure 3. The upper chart, A, shows the incidence of tumors in relation to dose in the first experiment (CT 620) as plotted from the values recorded in the last column of table 1. The results are the same

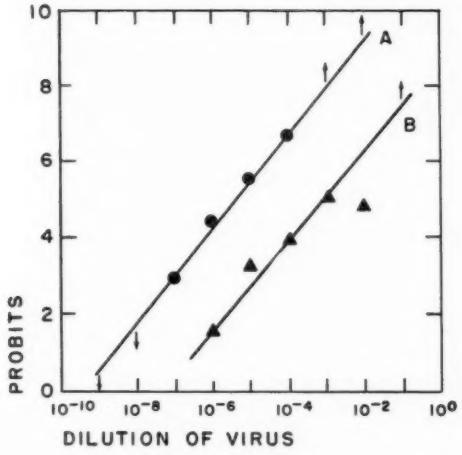


TEXT-FIGURE 3.—The *upper* chart, A, shows the incidence of tumors (in percent) in relation to dose in the initial experiment (CT 620) for the production of tumors. The *lower* chart, B, shows the results of bioassays of the extracts of experimental tumors induced with different doses of the standard virus (see text).

as those shown in probit form in text-figure 1. The "effective dose 50" (ED50) is seen to be at dilution $10^{-5.4}$. The lower chart, B, shows the results of bioassays of the tumor-tissue extracts. The open circles represent the potency estimates for tumors collected in the initial experiment (CT 620). The closed shaped points represent the supplementary tumors collected in subsequent experiments (see table 2B). The dash line indicating the limit of the bioassay method is an average value for the variable limit observed in different experiments, which was dependent upon the level of chicken-lot sensitivity. The points plotted below this line in the zone labeled "negative" represent statistical variables, also, rather than an absolute absence of the virus in each instance; *i.e.*, they were dependent upon the level of sensitivity of the most susceptible individual which chanced to be included in the particular chicken group used for the test. The "negative" results, accordingly, represent amounts of virus which were "less than could be detected" under the optimal technical conditions of the experiments. The level of relative potency of the standard is indicated by the arrow at 100 percent.

The potencies of the extracts of tumors produced by the 3 highest doses of virus (dilutions 10^{-1}, 10^{-2}, and 10^{-3}) do not, on a logarithmic scale, appear to be highly correlated with initiating dose, but are distributed about an average trend which appears to be approaching an upper limit asymptotically. This indicates that the concentration of extractable virus in the tumor tissues approaches a maximum value which is independent of initiating dose at the highest dose levels. It is of particular interest that these same doses produced a maximum (100 percent) incidence of tumors in test chickens. On the other hand, the dose range within which the tumor incidence was observed to drop progressively below 100 percent is also the range within which the log-percent potency estimates were found to be highly correlated with the log of the initiating dose of virus. All but 3 of the 17 tumors induced by doses equal to, or less than, ED50 yielded amounts of virus less than the standard by at least 2.5 $\log_{10}$ intervals, and less than the average maximal tumor yield by more than 3 $\log_{10}$ intervals. About half of them were less than the standard by 4 or more $\log_{10}$ intervals, and 4 out of the 17 (23.5 percent) failed to produce tumors at all under the technical conditions described. In view of the relatively large amounts of virus that may be lost during filtration through earthen filters (up to 1 or 2 $\log_{10}$ intervals of concentration), it is likely that a much larger percentage of the low-dose tumors would have been found to be "negative" on filtration.

The tumor-incidence (A) and relative-potency (B) data of text-figure 3 are plotted on arithmetic and logarithmic scales, respectively, and the trends of the plotted points are not directly comparable, although it is apparent that progressive decreases in both occurred within the same dose range. For better comparison of the respective trends, both types of data have been converted to the same units (probits) as shown in text-figure 4. The probit results for the former are the same as those included in text-figure 1. In order to convert the relative-potency data to proba-



TEXT-FIGURE 4.—Showing the similarity in probability units (probits) of the curves relating (A), the incidence of tumors, and (B), the potency of extracts, with the initiating dose of standard virus (see text).

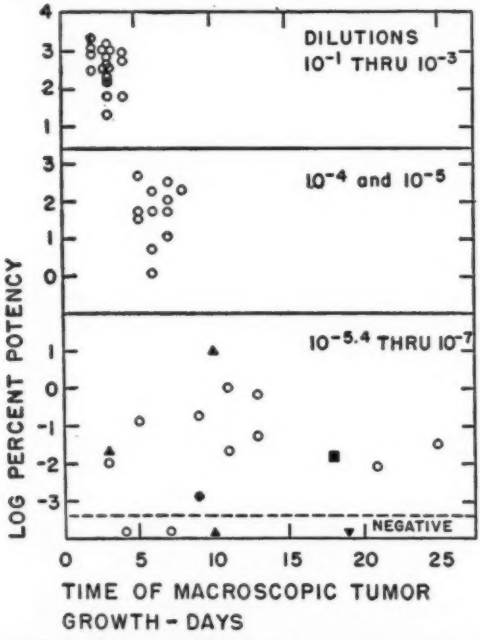
bility units (probits), the log-potency values (see table 2) were averaged within each dose group (using the median as the average, because of "negative" tumors at the lower dose levels), and a correction was applied for adjusting the average log-potency value for the highest dose (dilution 10^{-1}) to 2.00 (antilog = 100 percent). The same correction was then applied to all average log-potency values and the antilogs were found and converted to probits. The results are shown by the triangular points of text-figure 4. It is seen that with the exception of the average value at dilution 10^{-2}, the individual estimates for which varied over an unusually wide experimental range (see text-fig. 3B and table 2), the trend of the plotted points is essentially parallel to that observed for the tumor incidence data. It is apparent, therefore, that the two different types of biological variates are related to each other (*i.e.*, to the same underlying biological mechanisms) and that both are a function of the initiating dose of virus. As indicated by the horizontal interval between the two curves, however, the percentage (probit) values for the different variates are not identical at a given initiating dose, but the decline of the extract potencies with decreasing dose begins at a higher (2.2 $\log_{10}$ interval) dose level. If these same trends are extrapolated to dilution 10^{-8}, the expected frequency of tumors is found to be only 0.05 percent (1.7 probits), and the amount of virus which could be extracted from tumors occurring with this frequency (*i.e.*, in response to dilution 10^{-8}) would be expected to be, on an average, just at the limit of detection by the methods described. Few, if any, of them would be expected to be filterable.

Since the average latent period is highly correlated with dose [see above; also reference (*1*)], it was to be expected that tumors produced by the weaker doses of virus would appear later than those produced by the

stronger doses and that the total time elapsing between inoculation of virus and excision of tumor would grow longer as the dose was decreased, The question therefore arises as to which is the more important factor in determining the amount of extractable virus, (a) the low dose of virus, *per se*, or (b) the longer time of development and growth of the tumor, which is in itself a function of dose. Carr (*3*) did not consider the factor of virus dose, although inoculums of widely different potencies were apparently involved in the group of experimental tumors which he studied. He concluded that the correlation which he observed was accounted for entirely by the length of time of the association between tumor, or virus, and host.

In order to obtain information on this question, the data were analyzed within dose groups to determine whether or not there was any association between extractable virus and (1) the time of macroscopic growth of the tumor (*i.e.*, the interval between the first macroscopic appearance of the tumor and its excision), or (2) the total time from inoculation of virus to excision of tumor.

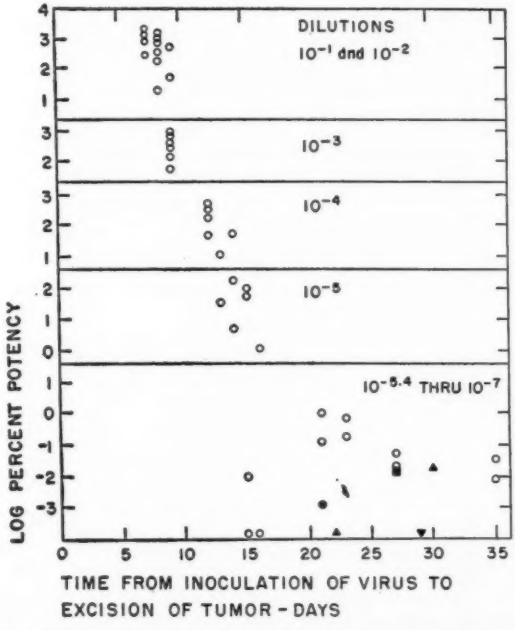
Preliminary analyses with respect to the time of macroscopic growth showed no apparent correlation within individual dose groups, and the data were therefore classified into coarser dose-interval groups to provide more data within categories for analyses of this type. The results over the whole dose range were thereby divided into high, intermediate, and low doses, as shown in text-figure 5. Even with this coarse classification,



TEXT-FIGURE 5.—Showing the lack of correlation between potency of extract and the time of macroscopic growth of tumor (see text).

no evidence was found of a significant correlation between the amount of extractable virus and the length of time the tumors had grown in their hosts after they were first detected on macroscopic examination. Some of the tumors produced by the lower doses grew quite rapidly after they had once appeared, and remained in their hosts for only a few days (*e.g.*, 3 to 5) after they were first detected. Little or no virus could be obtained from such tumors, although similarly rapid growing tumors induced by the strongest doses yielded maximal quantities of virus on extraction.

In analyses of the data of separate dose groups with respect to a possible association between extractable virus and the total time elapsing from inoculation of virus to excision of tumor, a suggestion of such an association was found. The data were therefore not combined into coarser groups within the dose range in which latent period and dose are correlated. They were combined, however, at the extremes of the dose range, in the regions in which dose and latent period are not correlated. The results of this analysis are shown in text-figure 6. The six tumors produced by dose 10^{-3} all happened to have been excised at a constant time (9 days), and no information on correlation between potency and time was obtained in this dose group. The trends of the plotted points in the 1st, 3rd, and 4th categories, however, are suggestive of an association within dose groups between extractable virus and total time from virus inoculation to excision of tumor. The data are not sufficient for conclusive proof of the apparent



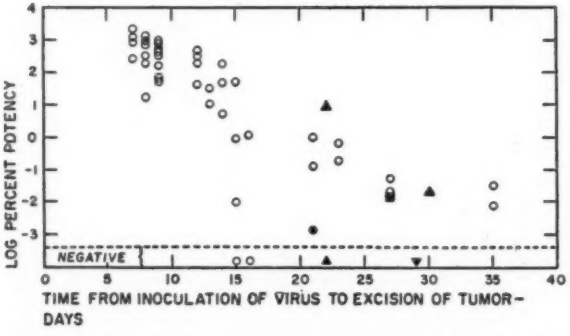
TEXT-FIGURE 6.—Showing the relationship between potency of extract and the total time elapsing between inoculation of virus and excision of tumor (see text).

trends, but they do suggest that the same factors which cause individual chickens to vary in their latent-period responses to a given dose may also be involved in determining the amount of virus that can be extracted from their tumors. The tumors resulting from doses equal to, or less than, ED-50, which have been grouped together in the lowest category of text-figure 6, show no correlation between extractable virus and the time elapsing after virus inoculation. The number of observations in this category is sufficient to establish this fact within the limits of time represented (15 to 35 days). None of the tumors of this category reached a size sufficient for study in less than 15 days, while all but one of them (which started to retrogress—see p. 319) had reached an adequate size (1.5 grams or larger) by the 35th day following virus inoculation.

Text-figure 7 shows the potencies of extracts in relation to total time of development and growth, irrespective of initiating dose of virus, which represents the form in which Carr (*3*) presented his results. The distribution of the plotted points is seen to be essentially the same as that found by Carr. Since the higher potency values are known to have all been obtained with high-dose tumors, whereas the lower values were all obtained with low-dose tumors, the results shown in text-figure 7 represent simply the plotting of one dependent variable (potency) against another (latent period, primarily), both of which are more fundamentally related to the controlled variable of these experiments, namely, virus-initiating dose.

Discussion

The dependence on initiating dose of the amount of virus extractable from Rous sarcomas could explain many of the bizarre and unaccounted for findings reported by earlier investigators. For example, Gye and Purdy (*6*) noted a rough correlation between tumor type (*e.g.*, morphologic characteristics, rate of growth, degree of malignancy) and the potency of extracts prepared from the tumors. They were able most of the time to prepare fairly potent filtrates by selection of the most malignant and the



TEXT-FIGURE 7.—Showing the relationship between potency of extract and total time from inoculation of virus to excision of tumor, disregarding initiating dose of virus (see text).

most rapidly growing tumors, but they were not always successful, because "strangely unaccountable exceptions" sometimes occurred. Rous and Murphy (*14*) also found that, whereas the strongest filtrates were obtained from highly malignant and rapidly growing tumors, not infrequently "filtrates prepared from malignant material under the best conditions proved entirely innocuous." Cramer and Foulds (*12*) stated that it was "impossible to predict" whether a particular Rous tumor would yield a completely inactive filtrate. As pointed out above, some of the low-dose tumors grew relatively rapidly once they had appeared. They were also indistinguishable, grossly, from many of the high-dose tumors, yet they yielded little or no virus on extraction. Another puzzling phenomenon reported by many of the earlier investigators, but no longer encountered in work with the stronger virus preparations of the present time, was the "temporary disappearance of filterability of tumors" [*cf.* (*15*, *16*) for discussions and review]. This phenomenon was manifested by the failure to recover virus in filtrates of tumors, sometimes for a period of several months, although the tumors could be maintained in the laboratory during this time by serial transplantations of tumor tissue into chickens (*11*, *15*, *16*). Eventually, however, the tumor passage material would again become filterable. In contrast with the stronger virus suspensions obtainable at the present time (by means of differential centrifugation and concentration in an ultracentrifuge) the earlier investigators used either filtrates of fresh tumor tissue or extracts of desiccated tissue to insure that their suspensions would be free of living cells. Both these latter types of materials are considerably weaker than unfiltered extracts or ultracentrifuged concentrates [(*5*, *11*, *12*, *17*) also, observations in this laboratory]. The lower relative potencies of the earlier virus inoculums is further substantiated by the fact that, not infrequently, one or more of the individual chickens in the small test groups (2 to 8) failed to develop tumors with the strongest suspensions available [*cf.* (*1*)]. The potencies of the preparations used for serial passage of the virus were, therefore, probably comparable with the standard materials of the present series (dilutions 10^{-5} to 10^{-4}), which produced tumors in only 70 to 95 percent of the inoculated chickens. The latter materials contained only about 2.5 to 25 ED50 units of virus per inoculum. Considering the relatively wide variability among individual chickens of a common lot in their susceptibilities to the Rous sarcoma virus (corresponding to about 3.5 $\log_{10}$ intervals of virus concentration between extremes—see S-shaped curve of text-figure 3A), as well as the fact that the average susceptibilities of chicken lots may vary widely [over a range corresponding to about 2 $\log_{10}$ intervals of virus concentration—see (*1*)] from time to time (*10*), it is not difficult to account for periodic absences of detectable virus in tumor-tissue filtrates on a basis of the present quantitative findings, and small samples of passage chickens taken during resistant phases of variation in chicken-lot susceptibility. As pointed out by Duran-Reynals and Freire (*2*), the age of the host chicken may also be of considerable importance in determining the filterability or nonfilterability of tumors.

A final point of interest regards the more general implications of the results. It has been generally assumed that tumors having a viral etiology should yield demonstrable virus on extraction, and that failure to demonstrate virus in filtrates of tumor-tissue extracts is justification for their classification as nonviral tumors. The present findings show decisively that this is not a valid assumption, and that under certain reproducible experimental conditions even the most potent and most rapidly acting tumor virus known is capable of initiating tumors from which little or no virus can be recovered on extraction. Extrapolation of the observed quantitative relationships between initiating dose and extractable virus to a lower level of virus concentration which would be expected to produce tumors with an incidence of only 0.05 percent or less (comparable to the frequencies of some spontaneous tumors in laboratory animals), leads to the expectation that no virus would be demonstrable in filtrates of Rous sarcomas thus produced. This same phenomenon could also explain the failure to demonstrate virus in the original growths of other chicken tumors which arose spontaneously, but which yielded demonstrable virus after serial transplantation through several passages and the enhancement of malignancy produced thereby (*14*, *18*, *19*).

Summary and Conclusions

A quantitative study was made of the relationship between the amount of Rous sarcoma virus which could be extracted from experimental tumors and the amount of virus contained in the inoculums used for inducing them. The extractable virus was found to approach a maximal concentration in the tissues of tumors induced by relatively strong doses [about 250 or more "50-percent effective" (ED50) units], but to be highly correlated with the dose at lower initiating dose levels. Weak inoculums containing only 1 ED50 unit, or less, produced tumors from which relatively little or no virus could be recovered on extraction. Four out of 17 such "low-dose" tumors yielded no demonstrable virus under the optimal technical conditions used in the tests. It is concluded that the amount of extractable virus is primarily related to initiating dose, rather than secondarily through the influence of the time of development-and-growth, which is also related to dose. The general assumption by investigators in the field of cancer research that the absence of demonstrable virus in tumor-tissue extracts is justification for considering the tumors to be of "nonviral" origin, is shown to be invalid.

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α-Keto Acids in Tissue Culture. I. The Concentration of Pyruvic and α-Ketoglutaric Acids in the Ultrafiltrates from Horse Serum and Chick-Embryo Extract[1]

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Horse serum and chick-embryo extract are constituents of the standard stock medium used for the nutrition of certain cell strains in this laboratory (*1*). Sanford *et al.* (*2*) have shown that the protein-free ultrafiltrate of chick-embryo extract (when used with the nonultrafilterable portion of the horse serum) is equivalent to whole-embryo extract for growth of the strain 929 mouse fibroblast cells (*2*).

In continuation of the program to identify and estimate the substances in the medium available to the cell, the α-keto acids of the protein-free ultrafiltrates from the two stock constituents of the medium were examined. This report is concerned with the estimation of the quantities of pyruvic and α-ketoglutaric acids. These keto acids were deemed important because of their repute as factors in the intermediary metabolism connected with carbohydrate breakdown and as part of the chain joining amino acids and carbohydrates in the metabolic cycle, and also for their role in the B-avitaminoses. Furthermore, examination of the amino-acid content of our standard chick embryo extract–horse serum medium had revealed that several of the amino acids were present in small amounts (*3*, *4*). Inasmuch as it has been shown that in the diets of intact rats certain of the keto acids can substitute for their amino analogues with respect to growth (*5–11*), it appeared desirable to determine what levels of pyruvic, α-ketoglutaric, and oxalacetic acids were available to the mouse cells in culture as suspensions.

In addition, examination of the glycogen content of the strain 929 and strain 721 cells, grown for long intervals as suspensions *in vitro*, revealed low glycogen content (*12*). Teng *et al.* (*13*) reported a far greater synthesis of glycogen by tissue slices when pyruvate and glucose were both present in the medium than when either alone was supplied. In fact, they reported a net loss of glycogen when the initial glycogen of the liver slice was high and glucose alone or pyruvate alone was furnished. The question then arose as to the adequacy of the initial levels of pyruvate in our standard culture medium. If the level were found to be lower than in

[1] Received for publication March 8, 1955.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

plasma, replenishing the pyruvate to a more nearly plasma-like level might be considered. Our knowledge is limited, so that it is not known whether findings in tissue-slice studies are comparable to findings from studies with cell suspensions, particularly since the cells from which our experimental populations were derived had been grown in *in vitro* culture for generations. As a point of departure it has been hypothesized that they would be comparable.

Because the concentrations of pyruvic and α-ketoglutaric acids were found to be lower in the ultrafiltrates than one would suspect that they might be from the literature dealing with plasma and blood concentrations (*14–23*), and because oxalacetic acid was not found at all, data were obtained on whole blood and on the serum from which the ultrafiltrates were derived as well as on aliquots of comparable embryo extract before filtration, and such data are included in the study.

Certain solutions, obtained as the supernatant cell-free fluid after culture of cells in the fluid, were examined to see whether there was any change in the concentration of the identified α-keto acids in the surrounding medium during growth of the cells. Such supernatants are ordinarily referred to as "used" medium. The control medium incubated without cells was also examined. The examination of the supernatants raised more questions than it answered with respect to the keto-acid concentrations, so that portion of the work is being continued with only an illustrative result reported herein.

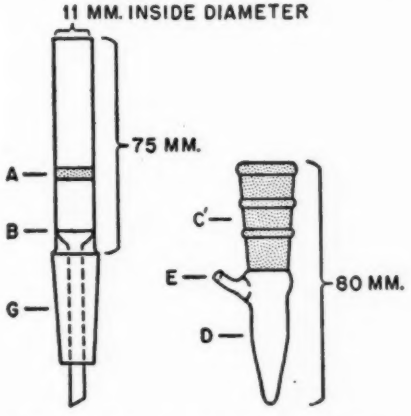
Experimental Procedure

The preparation of the ultrafiltrates using collodion membranes was the same as previously described (*2*). The α-keto acids were extracted from the ultrafiltrate as 2,4-dinitrophenylhydrazones and separated from each other by paper chromatography (*14*, *15*). Since the ultrafiltrates were protein-free, the usual deproteinization was unneeded, and smaller volumes of solution could be used, *i.e.*, one-tenth the usual Folin-Wu filtrate (*15*). After the evaporation of the solvent following the final partitioning between water and the chloroform-alcohol solvent,[3] the dinitrophenylhydrazones were taken up in pyridine. This solvent, though notoriously foul, was preferred to the buffered alcohol of Cavallini *et al.* (*14*) or the acetone solvent of Seligson and Shapiro (*15*). Both acetone and acetaldehyde, the latter as traces from the alcohol, readily form nitrophenylhydrazones (*24–28*), and it was feared there might be exchange with the keto acids under study. After the extracts were taken up in pyridine, they were spotted on Whatman #1 or #4 paper and the separation was carried out either in museum jars or in a large cabinet, the choice depending on the number of samples and length of time to be utilized. Following the separation of the hydrazones on paper, the paper was placed on an X-ray plate viewer, the spots were outlined with pencil in a darkened room, cut out, and placed in a small tube equipped with a fine-pored glass

[3] The extraction solvent of Seligson and Shapiro (*15*) was used.

frit (text-fig. 1). The paper was covered with 1 ml. *N* NaOH, the tube capped with Parafilm, and elution carried on for 10 minutes.[4] The tube and collecting base were then assembled and driven at 2,500 × g in a centrifuge. The extraction was repeated. Under these circumstances, practically the entire 2 ml. of the solvent came through into the vessel. The concentrations were then read in the Beckman spectrophotometer using 0.9-ml. volumes in micro quartz cups, sensitivity setting 1.0, slit 0.4, beam path 10 mm., and the hydrogen lamp as light source.[5] With 1 *N* NaOH set for 100-percent transmittance, 0.2 μg. of pyruvate and 0.3 μg. of α-ketoglutarate could be estimated with a reproducibility of 10 percent. However, it was usually unnecessary to work at such low concentrations, since the lowest of the quantities examined (those from certain of the horse-serum fractions) yielded 30 μg. of total keto acids, of which 5 μg. were α-ketoglutaric acid in the aliquot taken. The optimum quantity in 0.9 ml. used for the estimation was 1.5 μg. for pyruvic acid and 3.8 μg. for α-ketoglutaric acid. The alkaline solution of the pyruvyldinitrophenylhydrazone was read at 440 λ, and the α-ketoglutaryldinitrophenylhydrazone at 412 λ.

The following solvent systems were used for separating the dinitrophenylhydrazones on the paper: Solvent 1 (S1), as used by Cavallini *et al.* (*14*), *n*-butanol, ethanol, water (5:2:3 v/v). This solvent gave separation of the dinitrophenylhydrazones of oxalacetic, pyruvic, and α-ketoglutaric acid in a one-dimensional 24-hour run. Solvent 2 (S2) was the solvent preferred by Seligson and Shapiro (*15*), *n*-butanol saturated with 1 *N* sodium bicarbonate. The aqueous layer was drawn off after equilibration, placed in a container in the chamber, and the butanol



TEXT-FIGURE 1.—Centrifuge tube used in elution of the hydrazones from the filter paper. A is the fine-pored frit, B is the sealed-in collecting funnel, D is the eluate receiver, and E the air escape. G and C′ are 14/35 standard ground joints.

[4] The more usual procedure for eluting substances from the paper, by cutting the paper to a V-shaped point and allowing the solvent to flow over the hydrazone into the collecting vessel, uniformly gave low recoveries.

[5] In certain instances noted below the Coleman #14 instrument was used with the tungsten lamp.

layer used in contact with the paper. Solvent 3 (S3) was made up of *n*-butanol, isopropanol, and water (5:2:3 v/v). Like S1 and S5 this system yields a homogeneous solution. Solvent 4 (S4) was used for separating unchanged dinitrophenylhydrazine reagent and the hydrazones of acetone and acetaldehyde from those of the keto acids. S4 was *n*-propanol, light petroleum ether, toluene, benzene, and water (5:10:5:5:5 v/v). The organic layer was used on the paper. The α-keto-acid hydrazones under study moved very little in this less polar solvent, while the acetone and acetaldehyde derivatives moved just behind the solvent front with an Rf of 0.98. Solvent 5 (S5) was used in the later work primarily because it gave a more nearly reproducible distribution of the substance in the two spots assigned to pyruvyldinitrophenylhydrazone. S5 was *n*-butanol, ethanol, 2,6-lutidine, and water (45:20:20:15 v/v). Solvent 5 gave good separation of the two pyruvic acid spots from each other and from α-ketoglutaric acid, as well as from oxalacetic acid in a 16-hour run.

All the solvents could be used as received from commercial sources. The absolute ethanol contained 50 μg. of pyruvic acid per 100 ml.[6] The pyruvic acid could be removed by distillation from a solution of the dinitrophenylhydrazone. It was ordinarily more convenient to let this trace of pyruvic acid go through in the blank which was always run with each estimation. With the quantities in the usual extraction the blank would contain 5 μg. from this source. The commercial dinitrophenylhydrazine powder contained about 2.5-percent potassium sulfate. This inert salt presented no difficulty, and no attempt was made to remove it.

Because the ultrafiltrates required no deproteinization it was convenient to carry out an estimation of total keto acids directly on the unextracted filtrate. The information thus furnished was useful in indicating how much of the sample would be required for the extraction procedure and for subsequent chromatography on the paper used for separation of the pyruvic and α-ketoglutaric acids. This "direct" estimation referred to a pyruvic acid standard always gave higher values when calculated in terms of pyruvic acid than did the sum of the pyruvic and α-ketoglutaric acids separated on paper following the extraction, but, as already indicated, bore a sufficiently constant relationship to the postextraction and post-chromatographic values to be very useful as a guide in sampling.

The "direct" estimation was carried out as follows (*cf.* 27): Into a 30-ml. test tube was placed 1 ml. of the protein-free ultrafiltrate, which was then diluted to 10 ml. with water, and 0.4 ml. of the 0.2-percent dinitrophenylhydrazine in 2 *N* HCl added. The mixed solution was allowed to stand 30 minutes and 2 ml. of 5 *N* NaOH was added. The solution at first turned black, changing quickly to the red of the alkaline hydrazones. For the blank to which the instrument was set for 100-percent transmit-

[6] The alcohol contained a material that formed a hydrazone with the dinitrophenylhydrazine. This hydrazone gave the same distribution in the solvent extraction system as that from pyruvic acid. It gave the two spots under paper chromatography that were given by that from pyruvic acid; *i.e.* (with respect to Rf values) its alkaline red form gave the same absorption curve as that of pyruvic acid; and its absorption spectra, as the free hydrazone in chloroform, gave the curve identical with that from pyruvic acid.

tance, water was substituted for the ultrafiltrate and the rest of the procedure carried out as for the sample. The blank[7] likewise became black on alkalinization but changed immediately to light yellow. The concentrations were then read at 440 λ with the Coleman spectrophotometer, and the quantity was ascertained from a curve calibrated in terms of pyruvic acid only.

Sodium pyruvate was prepared by the method of Robertson (*29*) and the dinitrophenylhydrazone made from this by the method of Brady (*30*). α-Ketoglutaric acid was obtained from commercial sources and the dinitrophenylhydrazone made from this material (*30*). Two recrystallizations gave a product yielding a single uniform spot when carried as a control during the chromatography on the paper. Individual preparations were not used longer than 2 months owing to slow deterioration. Oxalacetic acid was obtained through the courtesy of Dr. Alton Meister, of the Laboratory of Biochemistry of this Institute. The dinitrophenylhydrazone made from oxalacetic acid was relatively unstable and was made as needed. We are indebted to Dr. Katherine Sanford, of this Institute, for aliquots of the ultrafiltrates from the horse serum and chick-embryo extract used in the experimental work.

Results

The concentration of pyruvic and α-ketoglutaric acids found in the ultrafiltrates from horse serum and in the serum furnishing these filtrates is shown in table 1. The samples were portions of those used in experimental work in cell growth previously reported by Sanford *et al.* (*2*). Part of these samples had been kept at 5° C. in the laboratory for some days before analysis. It was thought desirable to see how their concentrations compared with the concentrations obtained from freshly drawn serum. Data on keto acids in freshly drawn horse serum from 8 horses are shown in table 2. The keto-acid figures in this table were from tungstic-acid filtrates and are to be compared with the figures in columns 2 and 3 of table 1. The decrease with time after drawing the blood sample has been reported for the keto acids of other serums (*19–21*). The keto acids in the embryo-extract ultrafiltrate are shown in table 3. These ultrafiltrates were likewise part of those used in the cell-growth studies reported by Sanford *et al.* (*2*). Portions of the embryo extract furnishing the ultrafiltrate were not available for analysis, but comparable extracts were analyzed after removal of the protein with tungstic acid. The resulting data are given in table 4. The concentrations given for the extract are somewhat greater than those for the ultrafiltrate even though the extract was more dilute. The small decrease seems to be associated with the extra manipulation incident to preparation of the ultrafiltrates by the Simms' apparatus (*2*).

[7] Certain of the solutions examined contained phenol red. When present it gave minor interference. Its presence was corrected for by first determining the concentration of phenol red and putting the corresponding concentration in the blank.

TABLE 1.—*Concentration of pyruvic and α-ketoglutaric acids in pooled horse serum and its ultrafiltrate (mg. per 100 ml.*)*

Sample No.†	Whole serum		Ultrafiltrate	
	Pyruvic	α-Ketoglutaric	Pyruvic	α-Ketoglutaric
1	0. 4	0. 1	0. 4	0. 1
3	. 5	. 1	. 4	. 1
5	. 4	. 1	. 4	. 1
10	. 6	. 2	. 5	. 1

*The quantities have been rounded to the nearest whole fraction.

†These preparations were those previously examined for their filterable amino acids (3). Solvent 1 was used for the separation.

TABLE 2.—*The concentration of pyruvic and α-ketoglutaric acids in freshly prepared horse serum from individual horses**

Individual	Pyruvic acid (*mg./100 ml.*)	α-Ketoglutaric acid	Total
1	1. 0†	0. 25†	1. 3†
2	0. 85	. 20	1. 1
3	1. 15	. 3	1. 5
4	1. 1	. 3	1. 4
5	0. 8	. 2	1. 0
6	1. 0	. 2	1. 2
7	1. 1	. 2	1. 3
8	0. 9	. 2	1. 2

*Concentrations of pyruvic acid have been rounded to the nearest 0.05, *i.e.*, ± 2.5%; for the α-ketoglutaric acid they have been rounded to 0.01, *i.e.*, ± 5%.

†Concentrations were read for this table against a curve of pyruvic acid, calibrated also in terms of α-ketoglutaric acid, in order to read the figures recorded in the last column, *i.e.*, the last column does not consist of arithmetical addition of columns 1 and 2. Solvent 1 was used for the separation. Solvent 4 was used with these samples to separate the faster-moving, unknown spots from the faster of the pyruvate isomers.

TABLE 3.—*Concentration of pyruvic and α-ketoglutaric acids in embryo-extract ultrafiltrate (mg. per 100 ml.*)*

Sample No.†	Pyruvic acid	α-Ketoglutaric acid	Total "direct" as pyruvic
6	0. 2	0. 1	0. 4
7	. 15	. 05	. 35
8	. 15	. 05	. 30
10	. 2	. 1	. 40

*The quantities have been rounded to the nearest whole fraction.

†These preparations were previously examined for their filterable amino acids (3). Solvent 2 was used for the separation on paper. Aliquots were likewise run in solvent 3 with the same result.

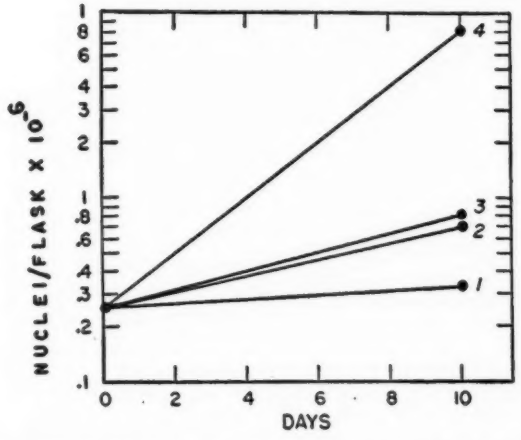
TABLE 4.—*Concentration of pyruvic and α-ketoglutaric acids in whole-embryo extract (mg. per 100 ml.*)*

Sample No.†	Pyruvic acid	α-Ketoglutaric acid
A	0.3	0.1
B	.35	.1
C	.3	.1
D	.4	.15
E	.35	.1
F	.35	.1

*These extracts were 1:1.25. When the above quantities are multiplied by the appropriate factor of 2.25, the resulting concentrations are in the general range of those found for serum.

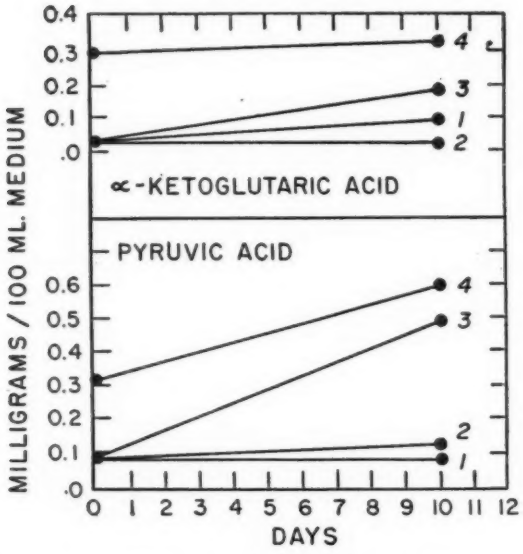
†Solvent 5 was used for the separation.

Portions of the ultrafiltrates were kept at 5° C. for 6 months under sterile conditions and were found to undergo a slow decrease in α-keto acids during this interval. However, both pyruvic and α-ketoglutaric acids were present at the end of this time. From the concentrations ascertained and the proportions of fluids used for the medium supplied to the cells, it can be calculated that the concentrations in the medium bathing the cells, when initially placed on them (see Discussion), would be no more than a third of that of plasma. The concentrations found by analysis of some of the experimental media before and after growth of the cells in them[8] with the corresponding growth curves and gross composition of the media are shown in table 5 and text-figures 2 and 3. The implications from these findings are being further explored.



TEXT-FIGURE 2.—Growth of the strain 929 mouse fibroblast cells in different media. Number of nuclei in the initial inoculum and final population. The initial inoculum was 260,000.

[8] We are indebted to Dr. V. J. Evans, of this Institute, for furnishing the used and fresh medium with the associated cell-growth data (*31*).



TEXT-FIGURE 3.—Concentrations of pyruvic and α-ketoglutaric acids in the media of text-figure 2 before and after growth of the cells, as noted in that figure. The source of the initial keto acids of all media except #4 was embryo extract; hence their graphs start from a common origin. Medium #4 contained horse serum which gave it an initially higher concentration of both acids.

TABLE 5.—*Compositions of the media examined for α-keto acids as recorded in text-figure 3 (composition of the media in parts by volume)*

	Medium number			
	1	2	3	4
Embryo-extract ultrafiltrate	20	20	20	20
Amino acid and vitamin mix, NCTC 102	40	40	40	40
Ovalbumin, 6.4%	0	20	20	0
Methocel, 0.5%, 4,000 cps.	0	0	20	0
Horse serum	0	0	0	20
Balanced saline	40	20	0	20

Discussion

In the paper chromatography of the dinitrophenylhydrazones the pyruvic acid yields two spots. Cavallini *et al.* (*14*) disregard the faster-moving of these and so report what appear to be low values for human serum. In agreement with Seligson and Shapiro (*15*), our studies indicate the faster-moving spot must be included as pyruvic acid. The two spots apparently must be attributed to geometric isomers similar to those studied for furfural dinitrophenylhydrazone by Bredereck (*32*) and Braddock *et al.* (*27*), and for the phenylhydrazone of acetaldehyde by Laws and Sidgwick (*25*). Also, it has been observed that the proportions

appearing in the two spots can be influenced by the relative acidity of the preparations before chromatographing as well as during the separation on paper, alkalinity favoring the preponderance of the faster-moving spot. Allowing the pyruvyldinitrophenylhydrazone to stand in the weakly basic solvent pyridine (used to take up the substance from the collecting vessel) gradually changes most of it to the faster-moving isomer. Both the serum and the embryo extracts, as well as their ultrafiltrates, show a fast-moving unidentified hydrazone beyond the faster-moving of the pyruvic acid derivatives and not far back of the solvent front. It is the presence of this fast-moving substance that is largely responsible for the total "direct" keto-acid concentration being greater than the sum of the α-ketoglutaric and pyruvic acids. It is likewise the presence of this fast-moving unknown substance that makes results too high for pyruvic acid when the method of Koepsell and Sharpe (*22*) is applied to the whole chloroform-ethyl alcohol extract following solvent removal.

During the chromatographic separation of extracts of the hydrazones using S4, a small amount of yellow material separates below the slower-moving pyruvate spot. This material does not give the red color of the keto hydrazones on treatment with sodium hydroxide in the routine extraction for quantitative estimation. When the separation is made using S5, it is included in the slower-moving of the pyruvyl derivatives. Apparently it is the 1-hydroxy-6-nitro-1:2:3:benzotriazole formed from the reagent by ring closure in the alkaline interval of the extraction process (*33*). Examination of a preparation of the triazole made by the method of Brady (*34*) indicated there was negligible interference with the estimation of pyruvate when readings were taken at 440 λ using the hydrogen lamp as light source. Furthermore, the triazole is part of the blank and is corrected for thereby. This fact is noted here because it is not generally realized that the triazole may be present in the usual extracts.

The standard medium used in this laboratory for the growth of certain cell strains consists of 40-percent balanced saline with 40-percent horse serum and 20-percent embryo extract. When used in these amounts, the concentration supplied to the cell even under the optimal circumstances would be about 30 percent of that in plasma. Whether or not this is a deficient level is not at present known.

The decrease to about half the original value in the concentrations of pyruvic and α-ketoglutaric acids shown in the serum (after standing overnight at 0° C. in contact with the cells) may indicate concomitant changes in concentration of certain of the amino acids taking part in the transamination reactions if the keto acids are not directly metabolized as such. Many questions arising from these and similar considerations are at present unanswered.

Summary

Horse serum, as prepared in this laboratory for use in stock medium for the culture of certain cell strains and its protein-free ultrafiltrate and the 9-day whole-chick-embryo extract and its ultrafiltrate likewise used on the stock cultures, have been examined for their content of pyruvic

and α-ketoglutaric acids. Freshly prepared horse serum was found to have approximately 1 mg. of pyruvic and 0.2 mg. of α-ketoglutaric acids per 100 ml. After the serum had stood overnight in contact with the blood cells at 0° C., had been gassed with CO_2, and put through the bacteriologic filter to sterilize, these concentrations decreased to about half. The protein-free ultrafiltrates from the prepared serum had the same concentration as the serum.

Chick-embryo extract made with 1.25 × the volume of balanced saline had approximately 0.35 mg. of pyruvic and 0.1 mg. of α-ketoglutaric acid per 100 ml. The protein-free ultrafiltrate had similar concentrations.

The standard medium of 40-percent horse serum plus 20-percent embryo extract could not contain more than 0.5 mg. per 100 ml. of pyruvic acid plus α-ketoglutaric acid. Exploratory studies on medium after cultivation of cells in it indicated that in media yielding good growth of cells the α-keto-acid level was quickly restored to a more nearly plasma-like level.

The α-keto acids were separated and estimated as the dinitrophenylhydrazones by solvent extraction and paper chromatography. By this procedure no oxalacetic acid was detected in the ultrafiltrates.

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ANNOUNCEMENT

Applications for Grants in Cancer Research

Acting for the American Cancer Society, the Committee on Growth of the National Academy of Sciences–Research Council is accepting applications for grants-in-aid for cancer research in the United States. Applications received before October 1 will be considered during the winter and grants recommended at that time become effective on July 1, 1956. *Investigators now receiving support will be notified regarding application for renewal.*

The Committee feels that an understanding of cancer depends upon a deeper insight into the nature of the growth process, normal and malignant. Therefore, the scope of the research program is broad and includes, in addition to clinical investigations on cancer, fundamental studies in the fields of cellular physiology, morphogenesis, genetics, virology, biochemistry, metabolism, nutrition, cytochemistry, physics, radiobiology, chemotherapy, endocrinology and carcinogenesis. The Committee is particularly interested in encouraging research in the epidemiology of cancer.

Application blanks may be obtained from the *Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N. W., Washington 25, D. C.*

Journal of the National Cancer Institute, Vol. 16, No. 1, August 1955

U. S. GOVERNMENT PRINTING OFFICE: 1955

The Journal of the National Cancer Institute is published bimonthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Six issues, beginning with number 1 in August, comprise each yearly volume. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

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The printing of this publication has been approved by the Director of the Bureau of the Budget on 18 August 1954

UNITED STATES GOVERNMENT PRINTING OFFICE, WASHINGTON : 1955

For sale by the Superintendent of Documents, U. S. Government Printing Office Washington 25, D. C. - Price per copy varies with each issue.

Subscription price per year in the United States, Canada, and Mexico, $7.50; Foreign, $9.25.

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